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Four conferences on interpretation of environmental data were conducted by the American Statistical Association in 1986 and 1987 under a cooperative agreement with the Statistical Policy Branch, OPPE. This article presents a summary of the first of these conferences held May 5-6, 1986.

Eight papers were presented by a variety of speakers assessing the effects of mixtures of toxicants.

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PREFACE

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This volume is a compendium of the papers and commentaries that were presented at the first of a series of conferences on interpretation of environmental data conducted by the American Statistical Association and the U. S. Environmental Protection Agency's Statistical Policy Branch of the Office of Standards and Regulations/Office of Policy, Planning, and Evaluation.

The purpose of these conferences is to provide a forum in which professionals from the academic, private, and public sectors can exchange ideas on statistical problems that confront EPA in its charge to protect the public and the environment through regulation of toxic exposures. They provide a unique opportunity for Agency statisticians and scientists to interact with their counterparts in the private sector. →con't to BDs

The holding of a research conference and preparation of papers for publication requires the efforts of many people. Gratitude is expressed to the ASA Committee on Statistics and the Environment which was instrumental in developing this series of conferences. Thanks are also owed to members of the ASA staff and, particularly, Ede Denenberg, who supported the entire effort. Although there was no provision for a formal peer review, thanks are also due to the reviewers who assessed the articles for their scientific merit and raised questions which were submitted to the authors for their consideration.

The views presented in this conference are those of the individual writers and should not be construed as reflecting the official position of any agency or organization.

Following the first conference on "Current Assessment of Combined Toxicant Effects," in May 1986, a second was held in October 1986 on "Statistical Issues in Combining Environmental Studies," from which a proceedings volume will also be published. The subject of the next conference, scheduled for May 1987, will be "Sampling and Site Selection for Environmental Studies."

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American Public Health Association

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MODELING THE JOINT ACTION OF TOXICANTS: BASIC CONCEPTS AND APPROACHES

Ralph L. Kodell, National Center for Toxicological Research

Introduction

The problem of modeling the joint action of drugs and environmental toxicants has seen a resurgence of interest recently, due to a heightened awareness of the need to protect health and environment, and the attendent regulatory considerations. The assessment of combined toxicant effects falls into the general framework of a mixture problem. There is a body of literature that deals with finding optimal mixtures of various components through the use of response surface methodology (Cornell, 1981). This approach has been used successfully, for example, to describe the effects of cancer chemotherapy treatments (Carter et al, 1984). In general, however, the assessment of mixtures of agents such as drugs and pesticides has tended to follow a more specialized approach (Kodell and Pounds, 1985). Most current efforts to study this type of joint action are based on the seminal work of practitioners such as Bliss (1939), Gaddum (1949), Hewlett and Plackett (1950), Finney (1952), and Loewe (1953). In drug development, the interest lies both in enhancing efficacious joint effects and in limiting toxic joint effects. In pesticide development, the interest lies in enhancing toxic effects to a targeted population, while limiting those toxic effects to untargeted populations. This is illustrated in Figure 1. In addition, it is important to know of any inhibitory effects of one beneficial drug or pesticide on another.

Generally speaking, in modeling the joint toxic action of agents administered in combination, the toxic endpoint produced by individual agents is known, and the objective is to determine whether the joint toxic action of two or more agents is in some sense "additive," as opposed to being "synergistic" or "antagonistic." In addition to basic research and development considerations, this has application in determining acceptable levels of exposure to environmental toxicants. Various scientific disciplines are involved, including biostatistics, pharmacology, toxicology and epidemiology.

Joint Action Nomenclature

In looking into the problem of investigating the joint action of toxicants, one immediately senses a lack of consistency among investigators with respect to the nomenclature used to characterize various types of joint action. For example, some authors use the term "synergism" very loosely to describe any enhanced joint effect, while others use a term "potentiation" to describe certain types of enhancement and synergism to describe others. The term "additivity" implies the absence of synergism to some, but is a special case of synergism to others. Berenbaum (1977) has described the inconsistent terminology surrounding synergism quite succinctly, although a bit harshly: "Synergy, however, is a topic on which confusion reigns. The relevant pharmacological literature is often obscure (some papers, indeed, are models of incomprehensibility) and is profusely littered with technical terms that are not always clearly defined. Several different terms are used to describe the same phenomenon and the same term means different things to different authors."

While clearly there is no consensus with respect to joint action nomenclature, there does seem to be a tendency to classify various types of joint action into either of two broad categories, namely, "interactive" and "noninteractive" action. Under the latter category, the concepts of "addition" and "independence" underlie various null models of joint action (Table 1). To the pharmacologist and toxicologist, the concept of addition or "additivity" can imply something about either the doses (concentrations) or the responses (effects) of toxicants acting together. To the biostatistician, addition of doses is in line with the concept of "similar action," whereas addition of responses is related to the notion of "independence" of action. To the epidemiologist, the concept of additivity relates only to the responses of jointly acting toxicants, and stems from the notion of independence of action. The epidemiologist includes the concept of "multiplication" of responses as a form of noninteractive joint action, in the sense that it can be interpreted as a type of independence of action. Table 2 gives a cross-classification of basic concepts by scientific disciplines.

TABLE 1. Concepts and nomenclature associated with the broad classifications of non-interactive and interactive joint action.

Noninteractive Action		
Addition		Independence
Concentration	Response Addition	Response Multiplication
Similar Action		Response Independence
Interactive Action		
Synergism		Antagonism
Potentiation		Inhibition
Enhancement		Attenuation
Supra-Addition		Infra-Addition

In the category of interactive joint action are included the various departures from additive and independent joint action. These interactions are often classified as either "synergistic" or "antagonistic," although increased effects are sometimes described as exhibiting "potentiation" or "enhancement" rather then synergism, and decreased effects as exhibiting "inhibition" or

TABLE 2. Concepts of noninteractive joint action, categorized by scientific disciplines in which they are used. Cell entries represent terms or notions within each discipline that are commonly used to describe the concepts of noninteraction. An empty cell implies that the discipline does not embrace the concept.

Null Model / Discipline	Concentration Addition	Response Addition	Response Multiplication
Toxicology/ Pharmacology	Additivity	Summation	
Epidemiology		Additivity	Multiplication
Biostatistics	Simple Similar Action	Unconditional Independence	Conditional Independence

"attenuation" rather than antagonism (Table 1). Numerous other terms have been used to describe interactive joint action, including supra- and infra-addition, super- and sub-addition, hyper- and hypo-addition and hyper- and hypo-multiplication.

Null Models for Noninteractive Joint Action

The primary focus of this paper will be on null models of concentration and response additivity as applied in a pharmacological / toxicological context. These models and concepts will be discussed initially. Following this, a less-detailed discussion of the additive and multiplicative models of relative risk employed in epidemiology studies will be given.

The basic approach to modeling the joint action of two (or more) toxicants is founded on tolerance distribution theory. That is, individuals are presumed to have varying degrees of tolerance to a particular toxicant, thus implying a probability distribution of tolerances. Dose-response models are formulated without attempting to identify specific underlying mechanisms of action of the toxicants under study. Pharmacological foundations for joint action studies are often attributed to Gaddum (1949) and Loewe (1953, 1957), while biostatistical modeling has been developed by Bliss (1939), Finney (1952, 1971), Hewlett and Plackett (1950, 1959), Plackett and Hewlett (1948, 1967), Hewlett (1969), Ashford (1958) and Ashford and Smith (1965). There has been some attempt to formulate more refined models in terms of their biological basis. For example, Ashford and Cobby (1974) developed a class of joint action models based on receptor theory and the law of mass action, following work by Plackett and Hewlett (1967) and citing the early work of Gaddum (1936). This work was followed-up by Ashford (1981). Although there has been some application of this theoretical approach (e.g. Chou and Talalay, 1983; Svensgaard and Crofton, 1985), virtually all practical investigations of joint toxic action have followed the tolerance distribution approach.

As alluded to above, generally the dose-response models that have been formulated for noninteractive joint action are based either on concentration addition or on response addition, or at least they include these types of joint action as special cases. Among the authors who have adopted the concept of concentration addition in modeling noninteractive joint action are Smyth et al. (1969), Casarett and Doull (1975), Piserchia and Shah (1976), Berenbaum (1977), Eby (1981), and Unkelbach and Wolf (1984). Among those who have modeled noninteractive joint action on the basis of response addition are Webb (1963), Holtzman et al. (1979), Wahrendorf et al. (1981), Ozanne and Mathieu (1983) and Machado and Bailey (1985). Authors who have modeled on the basis of both concentration addition and response addition include Broderius and Smith (1979), Shelton and Weber (1981), Chou and Talalay (1983), Kodell and Pounds (1985), Christensen and Chen (1985), and Chen et al. (1985). The terms "concentration addition" and "response addition" were introduced by Shelton and Weber (1981). Their idea of response additivity is slightly more general than its use in this paper. Loewe (1953) used the terms "iso-addition" and "hetero-addition" to describe a broad concept of concentration addition and a narrow concept of response addition, respectively. Steel and Peckman (1979) introduced the notion of an "envelope of additivity" that is bounded by Loewe's iso- and hetero-additivity.

Concentration Additivity

Some of the principles and concepts that underlie concentration addition will be given prior to presenting a formal mathematical definition. Under the broad category of similar action, Bliss (1939), Finney (1971) and Hewlett and Plackett (1959) all expressed the principle that two toxicants have the same site of primary action, while Ashford and Cobby (1974) expressed the principle that both toxicants act at all the same sites. Hewlett and Plackett (1959) regarded similar action as meaning that the physiological effects leading to the response are additive. In this sense of additivity, they allowed for imperfect correlation of tolerances to the two toxicants. In the narrower sense of additivity used in pharmacology, the tolerances are completely positively correlated, but apparently one toxicant is not necessarily a simple dilution of the other (Hewlett and Plackett, 1959). In the narrowest sense of additivity (similar action) is the concept of concentration additivity (simple similar action) (Bliss, 1939; Finney, 1952; Hewlett and Plackett, 1959), in which one toxicant is simply a dilution of the other with respect to administrated dose. This concentration additivity is also characterized by the perfect positive correlation of the individual tolerances to the two toxicants (Finney, 1971; Hewlett and Plackett, 1959).

Let $P(d_i)$ denote the probability of a toxic response to concentration d_i of toxicant i ($i=1,2$) such that

$$P(d_i) = F_i(d_i) \quad ,$$

for some monotonic functions F_i ($i=1,2$). If one toxicant is a dilution of the other, then $d_1 = pd_2$, where p is the relative potency of toxicant 2 to toxicant 1. The probability of a toxic response to the combination of d_1 and d_2, assuming concentration addition, is

$$\begin{aligned} P(d_1+d_2) &= F_1(d_1+pd_2) \\ &= F_2(d_1/p+d_2) . \end{aligned}$$

The pharmacological approach to assessing concentration additivity has been through the use of isobolograms (Hewlett, 1969), which are plots of pairs of doses of the two toxicants that jointly give fixed levels of toxic response. The curve that represents a given constant response is called an isobole (Figure 2). Under the broad definition of additivity, these isoboles are straight lines, but they are not necessarily parallel. Under the narrow definition of concentration additivity, with perfect positive correlation of tolerances, these isoboles are parallel straight lines with slope equal to the negative of the relative potency.

The biostatistical approach to assessing concentration additivity has involved the fitting of dose-response models. As a simple illustration, consider the parallel line assay technique whereby a suitable linearizing transformation (e.g., probit), $F_i^{-1}(d_i) = \alpha_i+\beta_i \log d_i$, is used (Finney, 1971). Setting $\beta_1 = \beta_2$ yields $p = \exp[(\alpha_2-\alpha_1)/\beta]$. Another simple method is the slope ratio assay technique whereby $F_i^{-1}(d_i) = \alpha_i + \beta_i d_i$, $\alpha_1 = \alpha_2$ and $p = \beta_2/\beta_1$. The joint response to d_1 and d_2 is predicted using either F_1 or F_2 with estimated parameter values, and the goodness-of-fit of the model is assessed (Kodell and Pounds, 1985). Often models of greater complexity have been used (Hewlett and Plackett, 1959; Christensen and Chen, 1985).

Response Additivity

As above, some of the principles and concepts that underlie response addition will be given prior to presenting a formal mathematical definition. Under the broad category of independent action, Bliss (1939) and Finney (1971) expressed the principle that two toxicants have different modes of action, whereas Hewlett and Plackett (1959) and Ashford (1981) expressed the principle that the toxicants have different sites of action. Hewlett and Plackett (1959) modeled biological independence without assuming statistical independence. That is, their definition of independent action allowed for correlation of tolerances to the two toxicants. More narrowly, some early investigators (e.g. Gaddum, 1949) modeled independence of action in the sense of "absence of synergism," assuming perfect positive or negative correlation of tolerances. In the narrowest sense of independence is the concept of simple independent action (Bliss, 1939; Finney, 1971), which is also called response additivity. This response additivity is characterized by zero correlation of the individual tolerances to the two toxicants (Bliss, 1939; Finney, 1971; Ashford and Cobby, 1974).

With $P(d_i)$ as defined above, the probability of a joint toxic response, assuming response additivity, is

$$\begin{aligned} P(d_1+d_2) &= P(d_1) + [1-P(d_1)]P(d_2) \\ &= P(d_2) + [1-P(d_2)]P(d_1) . \end{aligned}$$

That is, the response to the second toxicant over and above that of the first is simply an added effect based on the proportion not responding to the first toxicant, and vice versa. Note that

$$P(d_1+d_2) = P(d_1) + P(d_2) - P(d_1)*P(d_2) ,$$

which corresponds to the probability of the union of statistically independent events. Although response additivity doesn't mean simply adding response probabilities, the last expression above indicates that if these probabilities are small, then the product, $P(d_1)*P(d_2)$, will not greatly influence the joint response. However, some authors have just added responses, without regard to their magnitude (Holtzman et al., 1979; Ozanne and Mathieu, 1983). This latter approach is equivalent to hypothesizing independent action with perfect negative correlation of tolerances.

The use of isobolograms to identify response additivity has not been popular, perhaps because of a lack of agreement as to the shape and location of isoboles. For example, Webb (1963) and Hewlett (1969) suggest conflicting shapes and locations of isoboles for response additivity. Indeed, Christensen and Chen (1985) demonstrated various shapes of isoboles under response additivity.

The biostatistical approach to assessing response additivity has involved the fitting of dose-response models. For example, a simple procedure has been to formulate $P(d_1+d_2)$ as $F_1(d_1) + F_2(d_2) - F_1(d_1)*F_2(d_2)$, for suitably chosen F_i (e.g., Kodell and Pounds, 1985). $P(d_1+d_2)$ is predicted from separately estimated $F_i(d_i)$ functions, and the goodness-of-fit of the response additivity model is assessed. Often, more general models of response additivity have been used (Hewlett and Plackett, 1959; Shelton and Weber, 1981).

Application of Concentration and Response Additivity

The setting of water quality standards for multiple contaminants is an example of an activity that requires either knowledge of or assumptions about the joint action of these contaminants. Citing insufficient information on mixtures of environmental contaminants, the Safe Drinking Water Committee of the National Research Council (1980) stated that estimates of toxicity from acute exposures will, out of necessity, have to be based on a nonconservative assumption of additivity. The Committee went on to cite the work of Smyth et al. (1969), which is based on concentration additivity, as pertinent.

With respect to carcinogenic effects from chronic exposure, the Committee favored response additivity, stating that to estimate quantitatively the cumulative carcinogenic risk of several carcinogens, the individual risks might be added. The Committee stated that this approach assumes that interactions are not present and that the risks are small enough so that adjustments for joint probabilities are not needed.

Let D_1 and D_2 denote exposure levels of toxicants 1 and 2, respectively, that correspond individually to an acceptable level of risk, R. To insure an acceptable level of risk, R, to a combination, d_1+d_2, of toxicants 1 and 2 under concentration additivity, then d_1 and d_2 must satisfy (Finney, 1971)

$$\frac{d_1}{D_1} + \frac{d_2}{D_2} \leq 1 .$$

Equivalently,

$$\frac{\pi_1}{D_1} + \frac{\pi_2}{D_2} \leq \frac{1}{(d_1+d_2)} ,$$

where π_1 and π_2 are the respective proportions of toxicants 1 and 2 in the mixture. Under response additivity, if R is an acceptable level of risk for a combination, d_1+d_2, of toxicants 1 and 2, then d_1 cannot pose an individual risk exceeding R_1 and d_2 cannot pose an individual risk exceeding R_2, where $R_1+R_2 \leq R$.

It should be noted that there is a case for which concentration addition and response addition are indistinguishable mathematically, i.e., their predicted joint responses are mathematically identical. This is the case of the one-hit model. Suppose that

$$P(d_1) = F_1(d_1) = 1 - \exp[-\lambda_1 d_1] ,$$

$$P(d_2) = F_2(d_2) = 1 - \exp[-\lambda_2 d_2] .$$

With a double logarithmic linearizing transformation, parallel lines with slope=1 are obtained, enabling estimation of λ_1, λ_2 and the relative potency, $p = \lambda_2/\lambda_1$, where $d_1=d_2p$. Thus, under an assumed concentration-additive joint response,

$$\begin{aligned} P(d_1+d_2) &= F_1(d_1+pd_2) \\ &= 1 - \exp[-\lambda_1(d_1+pd_2)] \\ &= 1 - \exp[-\lambda_1(d_1+\lambda_2/\lambda_1\ d_2)] \\ &= 1 - \exp[-\lambda_1 d_1 - \lambda_2 d_2] . \end{aligned}$$

However, assuming a response-additive joint response,

$$\begin{aligned} P(d_1+d_2) &= F_1(d_1) + F_2(d_2) - F_1(d_1)*F_2(d_2) \\ &= 1 - \exp[-\lambda_1 d_1] + 1 - \exp[-\lambda_2 d_2] - 1 \\ &\quad + \exp[-\lambda_1 d_1] + \exp[-\lambda_2 d_2] \\ &\quad - \exp[-\lambda_1 d_1] \exp[-\lambda_2 d_2] \\ &= 1 - \exp[-\lambda_1 d_1 - \lambda_2 d_2] . \end{aligned}$$

Thus the assumption of either concentration or response additivity leads to the same predicted mathematical joint response function. Of course, this is true also for a strictly linear dose response model, which is the limiting form of the one-hit model as the dose approaches zero.

Interaction

As indicated earlier, there is no clear consensus as to what constitutes "interaction" of drugs or toxicants. In a broad sense, several authors have expressed the concept that interaction is characterized by one agent's influencing the biological action of the other (Bliss, 1939; Hewlett and Plackett, 1959; Ashford, 1981). However, there is disagreement when this broad concept is made more specific. Plackett and Hewlett (1967) pointed out differences between their concept of interaction and that of Ashford and Smith (1965), quoting their definition of interaction from an earlier paper (Plackett and Hewlett, 1952) as follows: "[Drugs] A and B are said to interact if the presence of A influences the amount of B reaching B's site of action, or the changes produced by B at B's site of action; and/or reversely, with A and B interchanged." Plackett and Hewlett (1967) contended that Ashford and Smith's (1965) definition of "noninteractive" action included only simple similar action with complete positive correlation of tolerances and independent action with zero correlation of tolerances, whereas their own definition would include both similar action with incomplete correlation of tolerances and independent action with nonzero correlation of tolerances as noninteractive.

The use of isobolograms to characterize "interactive" departures from additivity has suffered from inconsistent nomenclature, as pointed out by Hewlett (1969). Interestingly, Hewlett (1969) reserved the term synergism to describe an enhanced effect when only one of two agents is active individually, using the term potentiation to describe an enhanced joint effect for two separately active agents. However, he described a decreased joint effect in both cases by the term antagonism. Also, Hewlett (1969) described the joint action of two agents that are separately inactive but jointly active as "coalitive." Figure 3 illustrates some commonly accepted isobolographic representations of interactive joint action.

With respect to attempting to refine characterizations of joint interactive effects, Loewe (1957) seemed critical of the role that biostatistics has played in this effort. He was probably correct, to the extent that he was saying that tolerance distribution models that depend on quantal response bioassay data for their resolution have limited ability to define basic biological mechanisms. Plackett and Hewlett (1967) commented on identifiability limitations of tolerance distribution models.

The Additive and Multiplicative Models of Relative Risk

Relative risk is defined as the ratio of the risk due to a causal agent in the presence of background risk factors to the risk due simply to background factors. The additive model of relative risk used in epidemiology studies corresponds to response additivity in pharmacology/toxicology studies. It is based on an approximation to a model of "unconditional" independence of events, wherein causal agents and background factors act independently of one anther (Rothman, 1976; Hogan et al., 1976). However, it corresponds also to a model of mutually exclusive (and therefore nonindependent) events (Kodell and Gaylor, 1986). Under the additive model of relative risk, the relative risk due to two agents in combination is simply the sum of their individual relative risks. More specifically, $RR_{12} = RR_1 + RR_2 - 1$. All departures from this model are characterized as either synergistic or antagonistic.

The multiplicative model of relative risk does not have a corresponding null model in pharmacology/toxicology studies. It is based on a model of "conditional" independence in a statistical sense, for an event space appropriately defined (Kodell and Gaylor, 1986), having arisen originally from the multiplication of attributable risks (Walter, 1976; Walter and Holford, 1978). As its name implies, under the multiplicative model of relative risk, the relative risk due to two agents in combination is simply the product of their individual relative risks. That is, $RR_{12} = RR_1 * RR_2$. Departures from this model are termed either synergistic or antagonistic.

Hamilton (1979) reviewed various measures of synergism that are employed with two-by-two tables of cohort data from epidemiology studies. All have been designed to detect departures from the additive and multiplicative models of relative risk. Investigators who have discussed or used both the additive and multiplicative models of relative risk are Kupper and Hogan (1978), Koopman (1981), Thomas (1981), Siemiatycki and Thomas (1981), Hamilton (1982) and Reif (1984), the latter three being concerned specifically with joint carcinogenic risk. Notably, Hamilton and Hoel (1978) have considered concentration additivity, response additivity, and response multiplication all in the same context, namely, that of joint carcinogenic risk.

Siemiatycki and Thomas (1981) formulated several examples of the additive and multiplicative models in the context of the multistage model of carcinogenesis. They also demonstrated a nonidentifiability aspect of these models, in that data can be consistent with a particular model even though the underlying conditions for that model are not met. Hamilton (1982) also discussed nonidentifiability aspects of his postulated multistage model for joint carcinogenicity. It should be noted that apart from theoretical considerations of nonidentifiability, simple two-by-two tables of epidemiologic cohort data, upon which many studies of interaction of disease risk factors are (of necessity) based, contain limited information about the joint action of these risk factors.

Discussion

The study of the joint action of agents administered in combination is a very difficult undertaking both conceptually and practically. Even though there is common ground among investigators of joint toxic action, there is also a great deal of inconsistency and disagreement in nomenclature and concepts. It is recommended that attempts to assess combined toxicant effects be kept as simple as possible, in light of the crude data generally available for such assessments. Investigators should be careful to define their own terms precisely and to fully understand the terminology of others. Terms such as additivity, independence, synergism, and antagonism should not be used loosely. As has been shown, departure from one type of additivity, say concentration additivity, might imply another type of additivity, say response additivity, rather than a synergistic or antagonistic form of interactive joint action (Table 3).

TABLE 3. Illustration of incorrect conclusions that can be reached if only one type of "additivity" is considered as a model of noninteractive joint action. The shape of the underlying dose-response curves governs the type of error that might be made.

Dose-Response	True Situation	Null Hypothesis	Incorrect Conclusion
Convex	Concentration Addition	Response Addition	Synergism
Convex	Response Addition	Concentration Addition	Antagonism
Concave	Concentration Addition	Response Addition	Antagonism
Concave	Response Addition	Concentration Addition	Synergism

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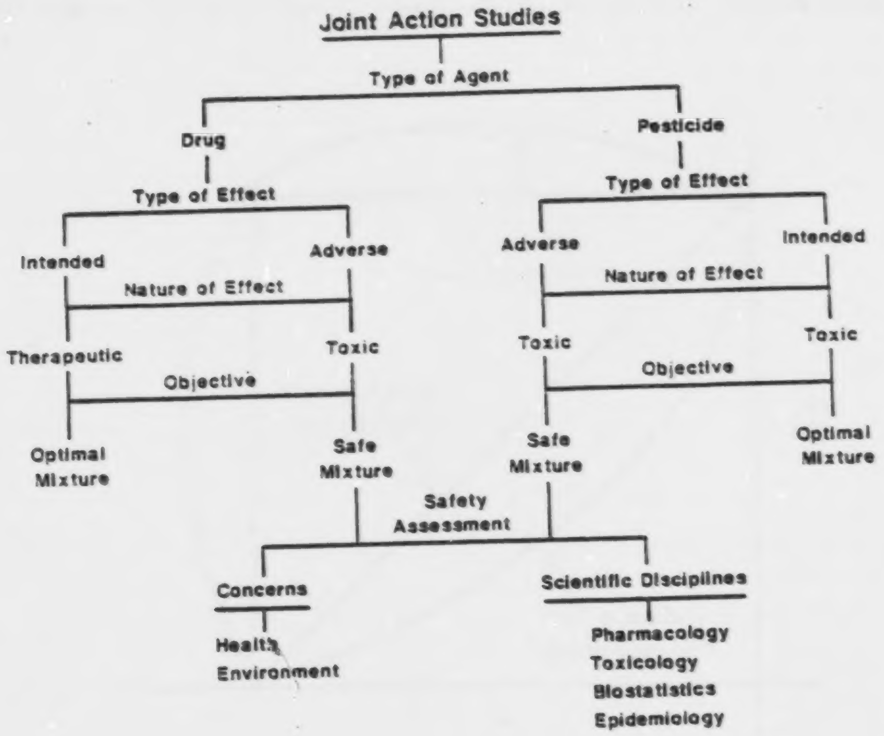
Joint Action Studies
Type of Agent
Drug
Pesticide
Type of Effect
Type of Effect
Intended
Adverse
Adverse
Intended
Nature of Effect
Nature of Effect
Therapeutic
Toxic
Toxic
Toxic
Objective
Objective
Optimal Mixture
Safe Mixture
Safe Mixture
Optimal Mixture
Safety Assessment
Concerns
Health
Environment
Scientific Disciplines
Pharmacology
Toxicology
Biostatistics
Epidemiology

Figure 1. Schematic representation of opposing objectives in joint action studies, along with concerns that motivate assessment of combined toxicant effects, and scientific discipline involved.

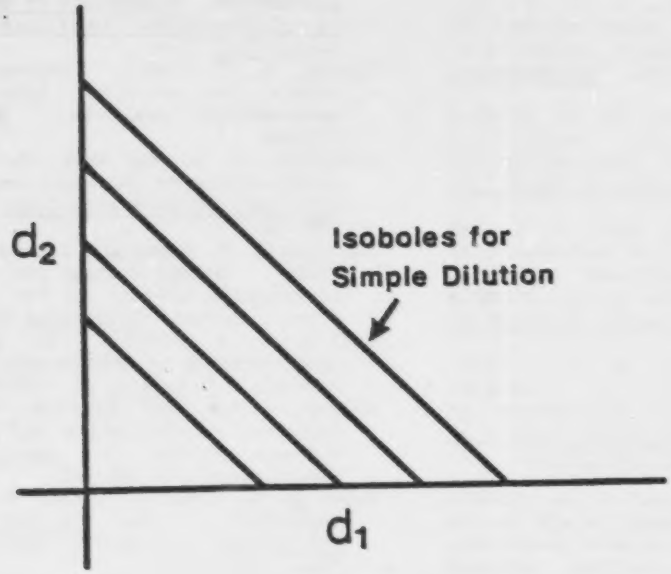
d2
Isoboles for
Simple Dilution
d1

Figure 2. Isobologram for assessing joint action. An isobole is a plot of pairs of doses of two toxicants that jointly give a fixed level of toxic response (e.g., 50%). For a simple dilution, isoboles for various response levels are parallel straight lines.

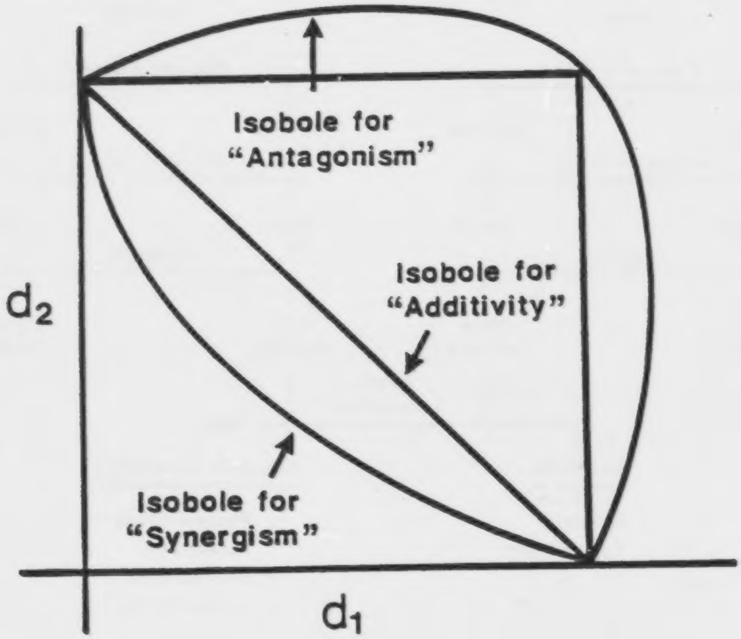
Isobole for
"Antagonism"
d2
Isobole for
"Additivity"
Isobole for
"Synergism"
d1

Figure 3. Isobologram depicting some commonly accepted, but not universally accepted, representations of concentration additivity, synergism, and antagonism. Isoboles for response additivity can lie anywhere within the square, depending upon the underlying dose-response curves.

USE OF THE MULTISTAGE MODEL TO PREDICT THE CARCINOGENIC RESPONSE ASSOCIATED WITH TIME-DEPENDENT EXPOSURES TO MULTIPLE AGENTS

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Introduction

In a review of multiple agent dose-response experiments, Filov et al. (1979) notes that the observed interaction effects are usually highly dose-dependent. As a result, such empirical tests of interaction as proposed by Hamilton and Hoel (1979), Machado et al. (1983), and Chen and Kodell (1986) performed at one set of dose levels may give very little information about interactions at another set of dose levels.

The "high" dose levels for joint effects are defined as the exposure values where statistically significant increases in cancer risk are observed in either epidemiological studies or cancer bioassays. For the most part, exposure to complex mixtures of agents in the environment is at "low" dose levels, i.e., at least three orders of magnitude below those at which a cancer response is observable in laboratory tests. As a result, empirical tests of interaction observed in bioassays give little insight into the effects of complex mixtures at environmental levels of exposure. To estimate effects at low dose levels, it is necessary to postulate an underlying theoretical construct for the carcinogenic process that can be translated into a mathematical dose-response model. Such a model will contain parameters describing various elements of the process. The joint effect of exposure to a complex mixture is determined by the way in which individual agents affect the parameters describing various elements of the process.

The agents in the complex mixture can interact to affect the process in a variety of ways. Chemical interaction between agents may create a different carcinogenic agent. An example of this in drinking water is the interaction of chlorine used as a bactericide with naturally occurring organic matter to form trihalomethanes (Bellar et al. 1974, Rook 1974). New compounds may form within the body as well. For example, nitrosation of certain compounds in fava beans by endogenous nitrite, when both are present in the gastric lumina, leads to the formation of a potent, direct-acting mutagenic nitroso compound (Yang et al. 1984).

Complex mixtures can also act to modify the exposed individual so that the dose at the site of action for one agent is dependent upon the exposure levels of the other agents in the mixture. Any event that affects the absorption, distribution, metabolism, or elimination of a compound will affect the level of that compound that is available to react with DNA or other target species. For example, simultaneous oral exposure to disulfiram (Antabuse) and inhalation exposure to ethylene dibromide can greatly increase the hepatocarcinogenicity of the latter. This increase is thought to be a result of the inhibition of acetaldehyde dehydrogenase by disulfiram, leading to the buildup of toxic metabolites of ethylene dibromide in the liver (Wong et al. 1982). Another example is exposure to cigarette smoke, which can induce the levels of cytochrome P450 and aryl hydrocarbon hydroxylase that metabolize polycyclic aromatic hydrocarbons (Conney et al. 1977), resulting in higher intracellular levels of reactive derivatives capable of forming adducts with DNA.

Another way in which biological interactions can enhance initiation is possible saturation of the enzyme systems responsible for the repair of DNA adducts, allowing some to go unrepaired and thus leading to mutation (Thilly 1983).

All such chemical-biological interactions are the result of reactions at many cellular sites with multiple molecules of the agents. As a result, mathematical models of the cancer response that depend upon such mechanisms would be nonlinear at low doses. For example, if two chemicals combined to form a carcinogenic agent, the rate of formation would be proportional to the product of the concentrations of the two chemicals. A linear reduction in the concentrations of the chemicals would thus result in a quadratic reduction in the formation of the carcinogenic agent.

The nonlinearity of the typical chemical-biological interaction strongly suggests that mechanisms of carcinogenicity that depend upon such interactions are only marginally important at environmental levels of exposure. Even so, any information about chemical interactions or exposure modification should be used in the formulation of a model of the joint effects of agents, if available, by estimating exposure at the cellular and molecular levels. For the mathematical model of the carcinogenic response discussed in the next sections, it will be assumed that the best available surrogate measure of dose at the site of action is used as the dependent variable.

Multistage Model

The most utilized quantitative model of the carcinogenic process is the simple multistage model described by Armitage and Doll (1954). This multistage model provides a satisfactory explanation of the power law for the age incidence of many forms of epithelial carcinoma. It also explains the time-dependent effects of variable exposures, including cigarette smoking (Armitage 1985). The multistage model is based upon the assumption that the carcinogenic process is a series of ordered, irreversible transformations in a single cell. After going through a fixed number of transformations, a cell is considered to be a tumor that will grow and be observed some time in the future.

If these transformations occur at the molecular level, it is reasonable to assume that a single molecule of an agent, if it enters the critical reaction, can cause the transformation from one stage to the next. Under this assumption, the probability of a transformation is linearly related to the degree of exposure at the molecular level.

For constant exposure to a single agent, the transformation rate from stage i to stage i+1 may be expressed as

$$(\alpha_i + \beta_i x), \tag{1}$$

where

α_i = background transformation rate,

β_i = transformation rate per unit of exposure, and

x = a constant that is directly proportional to the best surrogate measure of exposure at the site of action.

Assuming that there are a total of k stages and a fixed time w from the appearance of a cell in the kth stage to death by a tumor, the age-specific, agent-induced cancer death rate $[h(x,t)]$ is expressible as

$$h(x,t) = \prod_{i=0}^{k-1} (\alpha_i+\beta_i x)\ (t-w)^{k-1}/(k-1)!, \tag{2}$$

where

t = age attained.

The probability of death from a tumor by age t in the absence of competing mortality is simply

$$P(x,t) = 1-\exp -\int_w^t h(x,v)dv =$$

$$1-\exp - \prod_{i=0}^{k-1} (\alpha_i+\beta_i x)\ (t-w)^{k}/k! \tag{3}$$

The derivation of these results is presented clearly in the recent Armitage (1985) paper.

Generalization of Multistage Model to Account for Variable Exposure to Multiple Agents

The multistage model has previously been generalized to account for either exposure to multiple agents or variable exposure over time. Whittemore and Keller (1978) describe the complex equations that can be used to obtain estimates of risk under variable exposure conditions using the multistage model. Day and Brown (1980) give results for the case where observation continues after exposure ends. Crump and Howe (1984) derive an expression for the special case where one or two specified stages of the multistage process are assumed to be exposure-dependent, and exposure is taken to be a time-dependent step function. The multistage model has also been modified by Siemiatycki and Thomas (1981), Hamilton (1982), and Reif (1984) to account for exposure to multiple carcinogenic agents under constant exposure conditions.

To generalize the multistage model to account simultaneously for variable exposure and multiple agents, we start with a time-dependent exposure model. Following the approach taken by Whittemore and Keller (1978), we define the following variables:

$P_i(t)$ = probability that a cell is in the i^{th} stage at time t and

$\lambda_i(t)$ = transition rate from the i to the i+1 stage at time t.

The probability that a cell is in the i^{th} stage at time t(given that it is in the initial untransformed state at t = 0) can be described by the following set of simple differential equations:

$$dP_0(t)/dt = -\lambda_0(t)\ P_0(t) \qquad P_0(0) = 1$$

$$dP_i(t)/dt = -\lambda_i(t)P_i(t) + \lambda_{i-1}(t)P_{i-1}(t) \qquad P_i(0) = 0 \quad i = 1,\ldots,k-1$$

$$dP_k(t)/dt = \lambda_{k-1}(t)P_{k-1}(t) \qquad P_k(0) = 0 \tag{4}$$

To account for exposure to multiple carcinogenic agents, we define the transition rate to be

$$\lambda_i(t) = \alpha_i + \sum_{j=1}^{m} \delta_{ij}\ \beta_{ij}\ x_j(t), \tag{5}$$

where

α_i = background transition for i^{th} stage,

m = number of agents,

$\delta_{ij} = \begin{cases} 1 \text{ if the } j^{th} \text{ agent affects the } i^{th} \text{ stage} \\ 0 \text{ if otherwise,} \end{cases}$

β_{ij} = unit exposure transition rate for j^{th} agent on i^{th} stage, and

$x_j(t)$ = exposure to j^{th} agent at time t.

This formulation assumes that each of the molecules or produced radicals from all of the agents are acting independently of each other with regard to their probability of causing a cell transformation. This is reasonable when cell transformation probabilities for a single cell are very small, as would be the case when some individuals in the exposed population are free of the tumor in question.

Since the probability that a single specified cell will be transformed is very small, it follows that to a close approximation, $P_0(t) = 1$. Using this assumption, Whittemore and Keller (1978) showed that the preceding set of differential equations has the following approximate iterative solution:

$$P_0(t) = 1,$$

$$P_i(t) = \int_0^t \lambda_{i-1}(v)P_{i-1}(v)dv \tag{6}$$

In addition, we assume that

- The time required for a cell in its k^{th} transformed state to grow into a death-causing tumor is approximately constant and equal to the value w;

- The probability that a given cell will cause a tumor death is very small;
- An organ contains N cells of a specified type, each one of which is capable of causing a tumor death;
- N is very large;
- Each of the cells acts independently with regard to undergoing transformations and causing a tumor.

Then, the age-specific death rate associated with a specific type of tumor in a given organ may be expressed, to a close approximation, as

$$h(t) = N[dP_k(t-w)/dt] = N\lambda_{k-1}(t-w)P_{k-1}(t-w), \tag{7}$$

and the probability of death from that tumor by age t in the absence of competing risk is

$$P(t) = 1 - \exp - \int_0^t h(v)dv = 1 - \exp - \int_w^t N[dP_k(v-w)/dv]dv. \tag{8}$$

To illustrate how equations 5 through 8 can be used to estimate the risk associated with multiple-agent, time-dependent exposures, several simple examples will be presented in the following sections.

Example of Interaction Effects for Multiple Agents with Continuous Exposures at Constant Levels

For continuous, constant exposures, the transition rates are constants (over time) that are obtained from equation 5 by substituting x_j for $x_j(t)$. Using this notation, the transition rates have the form

$$\lambda_i(t) = \lambda_i = \alpha_i + \sum_{j=1}^{m} \delta_{ij}\, \beta_{ij} x_j, \tag{9}$$

and the possibility of a death from a tumor by time t is

$$P(x_1, x_2, \ldots, x_m, t) = 1 - \exp - \prod_{i=0}^{k-1} \lambda_i (t-w)^k/k! \tag{10}$$

At low environmental levels of exposure,

$$P(x_1, x_2, \ldots, x_m, t) \approx \left[\prod_{i=0}^{k-1} \lambda_i\right] (t-w)^k/k!, \tag{11}$$

where

$$\prod_{i=0}^{k-1} \lambda_i \approx \prod_{i=0}^{k-1} \alpha_i + \sum_{i=0}^{k-1} \left\{\left[\prod_{l=0}^{k-1} \alpha_l\right] \Big/ \alpha_i\right\} \sum_{j=1}^{m} \delta_{ij}\, \beta_{ij}\, x_{ij}. \tag{12}$$

since all higher-order exposure terms are approximately equal to zero.

A number of important implications follow from these results. When exposure to multiple carcinogenic agents occurs, each agent may affect one or more of the transition rates in one or more cell types. If two agents affect different cell types, their effect on the production of tumors will be independent if the appropriate mortality adjustment is made.

The probability of a tumor in this case is one minus the product of the probabilities that each agent does not cause a tumor. If the probability that each agent will cause a tumor is low, the probability that the joint exposure will produce a tumor is, to a very close approximation, equal to the sum of the probabilities that each agent causes a tumor. Where two agents act only on the same single stage of a cell type, the probability that joint exposure will produce a tumor is equal to the sum of the probabilities for each exposure. When the agents act on different stages of the same cell type, there is a multiplicative exposure effect term as well as the additive terms.

At high doses, the multiplicative exposure effect term can dominate the carcinogenic joint response, and the joint effect can be much greater than the sum of the individual effects. However, if both exposures are reduced by several orders of magnitude, the joint effect would be, to a very close approximation, equal to the sum of the individual effects. The same results hold when hundreds of compounds are combined. If each one is reduced three or more orders of magnitude, the deviation from additivity is not an appreciable relative amount. As a result, the multistage model predicts additivity at environmental exposure levels for almost all situations that would be routinely encountered.

The main exception to this rule is when one of the agents remains at a high level. In these cases, the incremental risk associated with exposure to low levels of an agent can be dominated by its multiplicative interaction with exposure to high levels of another agent. As a result, particular concern must be paid to agents that affect the same cell type as cigarettes, since cigarettes are the single deliberately uncontrolled carcinogen to which we are exposed at a high level in our environment.

To demonstrate the general premise that under multistage theory, an observed extensive synergistic effect in a multiple-agent bioassay does not imply a major departure from low-dose additivity, the following numerical example is given.

Simplest Multistage Model that Results in a Synergistic Effect

The simplest multistage model that results in a greater than additive effect arises from the assumption that each of two agents affects the transition rates of different single stages in the multistage process.

Thus, for two agents (m = 2), if the first agent affects the ith and the second, the jth stage and no other transition rates are affected, it follows that

$$\delta_{i1} = 1,\ \delta_{s1} = 0 \qquad s \neq i$$

$$\delta_{j2} = 1,\ \delta_{s2} = 0 \qquad s \neq j$$

Substituted into equation 5, this gives the result

$$\lambda_s = \alpha_s \qquad s \neq i,j$$

$$\lambda_i = \alpha_i + \beta_{i1}\, x_1$$

$$\lambda_j = \alpha_j + \beta_{j2}\, x_2$$

Assuming that competing mortality from causes other than the tumor under investigation is minimal at the termination of the experiment, the probability that a tumor will be observed may be expressed as

$$P(x_1,x_2)=1-\exp-[A_0(1+B_1x_1)(1+B_2x_2)], \qquad (13)$$

where

$$A_0 = \prod_{j=0}^{k-1} \alpha_j t^k/k!;\ B_1 = \frac{\beta_{i1}}{\alpha_i};\ \text{and } B_2 = \frac{\beta_{j2}}{\alpha_j}.$$

Consider a model of the form of equation 13 that has the following properties:

- One agent is twice as potent as the other,
- 0.1 of one agent and 0.2 of the other gives about a 9% response in a bioassay if each agent is given by itself,
- Responses at exposures of $1 \cdot 10^{-4}$ of the single agent values give a risk of $1 \cdot 10^{-5}$ for each agent singularly and $4 \cdot 10^{-5}$ for joint exposure to both agents, and
- The background risk is about $5 \cdot 10^{-6}$.

A numerical model that meets these conditions is

$$P(x_1,x_2)=1-\exp(-0.000005)(1+189{,}728x_1)(1+94{,}864x_2)$$

or $\qquad (14)$

$$P(x_1,x_2)=1-\exp-(0.000005+0.948640x_1+0.474320x_2$$
$$+89{,}991.8x_1x_2).$$

This model implies that to achieve a meaningful (i.e., doubling) joint exposure effect at low environmental doses, the joint experimental synergistic effect would have to be very large.

Two agents given together at levels of about 5% of the single-agent doses would produce about a 99% response, while the single-level doses given by themselves would yield about a 9% response. An interaction of this magnitude is unprecedented. This hypothetical situation is depicted in Table 1.

In the next section, implications concerning the ordering of exposure will be investigated.

Example of Interaction Effects when Multiple-Agent Exposures are not Continuous and Concurrent over Time

Variable and noncontinuous exposure patterns may be accounted for by treating the time-dependent exposures, $x_j(t)$, as specific step functions that allow equation 4 to be solved in a closed form. The following simple example illustrates this general approach.

Consider the case where exposure is to two agents ($m = 2$) with the following exposure patterns:

$$x_1(t) = \begin{cases} x_1 & s_1 < t < f_1 \\ 0 & \text{elsewhere} \end{cases} \qquad (15)$$

$$x_2(t) = \begin{cases} x_2 & s_2 < t < f_2 \\ 0 & \text{elsewhere} \end{cases}$$

where

s_1 = starting time of exposure to first agent,

f_1 = stopping time of exposure to first agent,

s_2 = starting time of exposure to second agent, and

f_2 = stopping time of exposure to second agent.

It is assumed that $x_1(t)$ affects the first stage only and that $x_2(t)$ affects the last or kth stage only. Under this assumption, the transition rates have the following time-dependent form:

$$\lambda_0(t) = \begin{cases} \alpha_0 & t < s_1 \\ \alpha_0 \beta_{01} x_1 & s_1 < t < f_1 \\ \alpha_0 & f_1 < t \end{cases}$$

$$\lambda_s(t) = \alpha_s \quad s = 1,2,\ldots,k-2 \quad 0 < t < \infty \qquad (16)$$

$$\lambda_{k-1}(t) = \begin{cases} \lambda^{(0)} = \alpha_{k-1} & t < s_2 \\ \lambda^{(1)} = \alpha_{k-1}+\beta_{k-1,2}x_2 & s_2<t<f_2 \\ \lambda^{(2)} = \alpha_{k-1} & f_2<t \end{cases}$$

Equation 16 is substituted into equation 6, and $P_{k-1}(t)$ is obtained from the iterative solution. This solution has the following form:

$$P_{k-1}(t) = \begin{cases} P^{(0)} = At^{k-1} & s_1 \geq t \\ P^{(1)} = At^{k-1}+A(\beta_{01}/\alpha_0)x_1(t-s_1)^{k-1} & s_1 <t<f_1 \\ P^{(2)} = At^{k-1}+A(\beta_{01}/\alpha_0)x_1[t-s_1)^{k-1} -(t-f_1)^{k-1}] & t>f_1 \end{cases} \qquad (17)$$

where

$$A = \prod_{j=0}^{k-2} \alpha_j/(k-1)!.$$

For $w = 0$, the age-specific rate defined in equation 7 is

$$h(t) = N\lambda_{k-1}(t)\ P_{k-1}(t). \tag{18}$$

Since $P_{k-1}(t)$ is functionally dependent upon s_1 and f_1 and $\lambda_{k-1}(t)$ is functionally dependent upon s_2 and f_2, it follows that $h(t)$, as defined in equation 16, is dependent upon the ordering over time of s_1, f_1, s_2, and f_2. For example, if $s_1 < s_2 < f_2 < f_1$, it follows that the products of $\lambda_{k-1}(t)$ and $P_{k-1}(t)$ have the time-dependent representation

$$h[x_1(t),x_2(t)] = \begin{cases} \lambda^{(0)}P^{(0)} & t \le s_1 \\ \lambda^{(0)}P^{(1)} & s_1 \le t \le s_2 \\ \lambda^{(1)}P^{(1)} & s_2 \le t \le f_2 \\ \lambda^{(2)}P^{(1)} & f_2 \le t \le f_1 \\ \lambda^{(2)}P^{(2)} & f_1 \le t \end{cases}$$

where the $\lambda^{(\cdot)}$ and $P^{(\cdot)}$ are defined in equations 16 and 17, respectively. A schematic representation of equation 19 that illustrates how the structural form of the age-specific rate is time-dependent is shown in Figure 1. Other structural relationships can be derived in the same manner for alternative orderings of the exposures.

To explore the effect of the timing of exposure on the interaction or synergism of the agents, we will estimate "relative risks" for the following situation. It is assumed that agent exposures were selected so that each of the transition rates is increased by a relative factor of Δ during the exposure interval. This implies that

$$\frac{\beta_{01}x_1}{\alpha_0} = \frac{\beta_{k-1,2}x_2}{\alpha_{k-1}} = \Delta. \tag{20}$$

Under this assumption, the relative augmented risk for the two exposures given together, as compared to the sum of the two given separately, can be derived. For most situations at environmental levels of exposure, this relative risk in the absence of competing risk may be expressed as

$$R^*(0,t) = \int_0^t \{h[x_1(v),x_2(v)]-h(0,0)\}\,dv \div \int_0^t \{h[x_1(v),0]+h[0,x_2(v)] -2h(0,0)\}\,dv. \tag{21}$$

The age-specific cancer rates for the four combinations of exposure depicted in Table 2 are derived in a manner analogous to the preceding example. These rates are then substituted into equation 21 and used to obtain the relative risks also depicted in Table 2.

The results obtained using this approach conform to one's intuitive sense of reasonableness. No synergism (i.e., effect greater than one) exists if the exposure that affects the last stage ends before the exposure that affects the first stage begins. Also, the greatest synergism exists when exposure that affects the first stage ends before that which affects the last stage starts. In this situation, the relative risk rises slightly from $1 + \Delta/3$ to $1 + \Delta/2$, as the number of stages increases from $k = 2$ to $k = \infty$. In contrast, the relative risk decreases rapidly from $1 + \Delta/4$ to 1 as the number of stages increases from $k = 2$ to $k = \infty$, for the situation in which both exposures are given during the same half of the time period. It is possible to derive comparable results for any set of assumptions about the stages affected and any step functions of exposure. However, the algebraic form may be very complex.

In the final section, the most important practical problem concerning joint exposure will be investigated — namely, how to cope with the potential interaction of cigarette smoke and other carcinogenic agents.

Joint Effect of Cigarette Smoke and Other Agents on Respiratory Cancer

As a first step in attempting to estimate how smoking cigarettes modifies the quantitative effect of other agents on cancer rates, it is necessary to develop a model for the effects of cigarettes alone. Ideally, we would use the combined data from as many sources as possible in such an endeavor. Unfortunately, the only data currently available in the open literature in a form amenable for fitting with a multistage model are found in the Doll and Peto (1978) paper; they are reproduced here as Table 3. It is recognized that a number of problems exist in using these data. Among the more important are the following:

- British cigarettes and/or smoking patterns are different from those in the United States.
- The data are in a form that results in a loss of information, since they are combined into various groupings rather than being presented for each individual.
- No information is given on rates after the cessation of smoking.

It is hoped that the availability of additional U.S. data and more complete data for the Doll and Peto (1978) cohort will eliminate these problems in the future. However, for the present we will fit the data given in Table 3 to various forms of the multistage model to illustrate the general approach for predicting the modifying effect of cigarettes on the cancer potency of other agents.

It is assumed that the 1st and kth stage of a k-stage model are affected by cigarettes. In addition, we assume that each individual in the cohort began smoking at age s_1 and continued until the end of the observation period. Also, we assume a constant lag or weighting time of length w, which will be estimated from the data. Under these assumptions, the equations for the transition rates may be expressed as

$$\begin{aligned} \lambda_0(t) &= \alpha_0 + \beta_{01}x_1(t) \\ \lambda_i(t) &= \alpha_i \qquad i = 1,2,\ldots,k-2 \qquad (22) \\ \lambda_{k-1}(t) &= \alpha_{k-1} + \beta_{k-1,1}x_1(t) \end{aligned}$$

where the number of cigarettes smoked per day has the functional form

$$x_1(t) = \begin{cases} x_1 & s_1 \leq t \\ 0 & \text{elsewhere.} \end{cases}$$

To incorporate a constant lag time of length w, we simply replace t with t-w. In addition, we can only estimate the ratio of the transition rates, which we denote with capital letters and equivalent subscripts. Using these two conventions, the age-specific death rate from respiratory cancer may be written as

$$h(x,t) = \begin{cases} A(t-w)^{k-1} & w \leq t \leq s_1+w \\ A(t-w)^{k-1} & \\ +[AB_{01}(t-s_1-w)^{k-1}+AB_{k-1,1}(t-w)^{k-1}]x_1 & s_1+w \leq t \\ +AB_{01}B_{k-1,1}(t-s_1-w)^{k-1}x_1^{\,2} & \end{cases} \qquad (23)$$

where

$$A = \prod_{j=0}^{k-1} \alpha_j/(k-1)!,\ B_{01}=\beta_{01}/\alpha_0,\ B_{k-1,1}$$

$$= \beta_{k-1,1}/\alpha_{k-1}.$$

The parameters A, B_{01}, $B_{k-1,1}$, and w can be estimated from the data in Table 3 using the maximum likelihood method. To do so, it is assumed that the observed number of respiratory cancer deaths in each cell has a Poisson distribution with mean h(x,t)PY, where PY is the total number of person-years observed for each cell. The parameter estimates that maximize the likelihood are shown in Table 4. The goodness of fit of the model is illustrated in Table 5. It is assumed that for each cell, s_1 equals 19.2, the average age at which people started smoking for the entire cohort.

The parameters k and w are highly negatively correlated, so that other estimates give almost as good a fit. Models that contain values for k and w that fall in the range shown below do not give a statistically significant worse fit at the 0.05 level, as measured by the log likelihood criteria, than the best fit shown in Table 4.

If $k=4$, then $13.9 < w < 23.3$

If $k=5$, then $4.8 < w < 22.2$

If $k=6$, then $0 < w < 14.2$

However, if $k < 3$ or $k > 7$, the fit is statistically rejected at the 0.05 level for all values of w. If the data on risks after the cessation of smoking were available, it is likely that only a k of 5 or 6 would fit the data; we would expect a short lag because risks fall quite quickly after smoking is stopped.

For the purpose of illustration, we will use our best-fitting model to predict the effect of smoking on the augmented risk associated with another agent. If it is assumed that a second agent affects the 1st stage of the multistage process, the transition rate for the 1st stage is expressed as

$$\lambda_0(t) = \alpha_0 + \beta_{01}x_1(t) + \beta_{02}x_2(t). \qquad (24)$$

For the case where $x_2(t) = x_2$ for all t, the age-specific rate under this added assumption has the form

$$h(x_1,x_2,t) = \begin{cases} A(t-w)^{k-1}[1+B_{02}x_2] & w \leq t < s_1 + w \\ A(t-w)^{k-1}[1+B_{02}x_2] & \\ + AB_{01}x_1(t-w-s_1)^{k-1} & \\ + AB_{k-1,1}x_1[1+B_{02}x_2](t-w)^{k-1} & s_1+w \leq t \\ + AB_{01}B_{k-1,1}x_1^{\,2}(t-w-s_1)^{k-1} & \end{cases} \qquad (25)$$

where

$$B_{02} = \beta_{02}/\alpha_0.$$

To obtain information about the parameter B_{02}, it is assumed that an animal bioassay is available. In terms of our previous parameters, the probability of response for the animal may be expressed as

$$P(x_2,t) = 1 - \exp - [A_0 + \frac{A}{k} B_{02}x_2(t-w)^k]. \qquad (26)$$

Of course, only the whole term, $(A/k)B_{02}(t-w)^k$, can be estimated from the quantal animal data alone; however, in conjunction with the human data, B_{02} may be estimated separately.

The augmented risk associated with continuous exposure to x_2 while smoking x_1 cigarettes per day from age s_1 to t under the assumption of no competing risk may be expressed as

$$P(x_2/x_1,t)=[1-\exp-\int_0^t h(x_1,x_2,v)dv]-$$
$$[1-\exp-\int_0^t h(x_1,0,v)dv]. \qquad (27)$$

For a low background rate, the augmented risk is, to a close approximation,

$$P(x_2/x_1,t)= \frac{A}{k} B_{02}x_2 \left\{1+x_1B_{k-1,1} \left[1- \left(\frac{s}{t-w}\right)^k\right]\right\}(t-w)^k \qquad (28)$$
$$s+w < t$$

To illustrate the general approach, we shall assume that the bioassay gave a linear term estimate of

$$\frac{A}{k} B_{02}(t-w)^{k} = 0.2.$$

Substituting this value and $w = 13.642$, $t = 70$, $s = 19.2$, and $B_{k-1,1} = 0.31044$ into equation 28 gives the numerical result

$$P(x_2/x_1, t=70) = 0.2x_2 \left\{1+0.31044x_1 \left[1- \left(\frac{19.2}{56.358}\right)^5\right]\right\}$$
$$= 0.2x_2 + 0.0618x_1x_2. \qquad (29)$$

Let us assume that without cigarette use the predicted risk is $1 \cdot 10^{-5}$ based only on the animal bioassay. This implies that x_2 equals $5 \cdot 10^{-5}$. Using equation 29, we calculate the augmented risk associated with the second agent alone in the presence of cigarette smoke for individuals who started smoking at age 19.2 and continued until death or age 70. These results are depicted in Table 6.

Thus, a person who smokes two packs a day increases his or her augmented risk by more than one order of magnitude. The interesting philosophical public health question arises: Does society have the responsibility for protecting an individual from a second agent that increases the involuntary risk by about $1 \cdot 10^{-4}$ if the individual smokes two packs a day, when the voluntary risk he or she assumes for smoking is about $1 \cdot 10^{-1}$ or three orders of magnitude higher.

In summary, the approach suggested here to adjust for cigarette use employs

- o Human data to estimate the effects of cigarettes,
- o Animal data to estimate the effects associated with the second agent, and
- o Multistage theory to predict the joint effects of cigarettes and the second agent in the absence of any actual joint exposure data.

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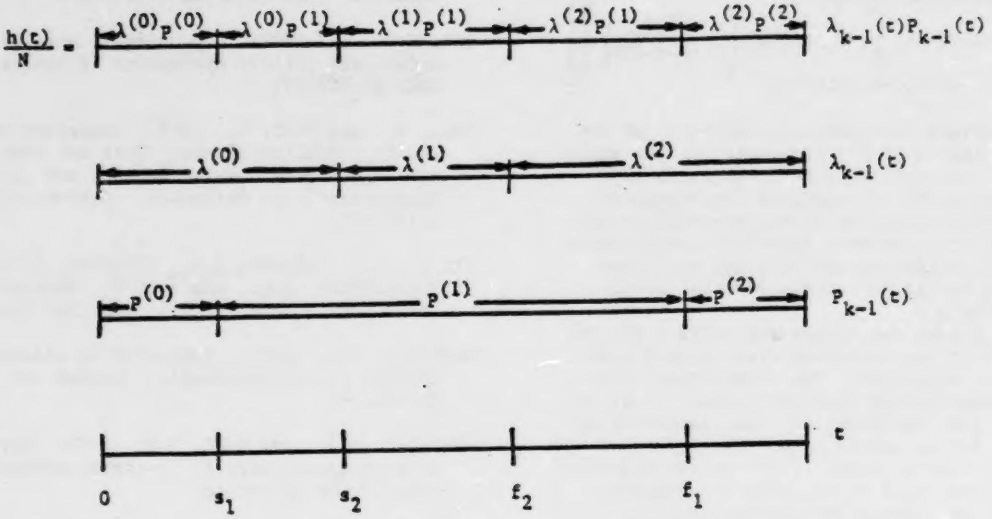
FIGURE 1

SCHEMATIC REPRESENTATION OF THE TIME-DEPENDENT,
AGE-SPECIFIC DEATH RATE SHOWN IN EQUATION 19


$\frac{h(t)}{N}$ =
$\lambda^{(0)}P^{(0)}$
$\lambda^{(0)}P^{(1)}$
$\lambda^{(1)}P^{(1)}$
$\lambda^{(2)}P^{(1)}$
$\lambda^{(2)}P^{(2)}$
$\lambda_{k-1}(t)P_{k-1}(t)$
$\lambda^{(0)}$
$\lambda^{(1)}$
$\lambda^{(2)}$
$\lambda_{k-1}(t)$
$P^{(0)}$
$P^{(1)}$
$P^{(2)}$
$P_{k-1}(t)$
t
0
s_1
s_2
f_2
f_1

NOTE: $\lambda^{(\cdot)}$ is defined in equation 16 and $P^{(\cdot)}$, in equation 17.

TABLE 1

BIOASSAY DESIGN

	x_1			
x_2	0	$1.05414 \cdot 10^{-5}$	$4.8743 \cdot 10^{-3}$	$1 \cdot 10^{-1}$
0	$\underline{0}$	$1 \cdot 10^{-5}$	--	$\underline{9.0503 \cdot 10^{-2}}$
$2.10828 \cdot 10^{-5}$	$1 \cdot 10^{-5}$	$4 \cdot 10^{-5}$	--	--
$9.7486 \cdot 10^{-3}$	--	--	$\underline{9.852 \cdot 10^{-1}}$	--
$2 \cdot 10^{-1}$	$\underline{9.0503 \cdot 10^{-2}}$	--	--	--

NOTE: This is the design required to estimate an interaction term large enough to double the risk over that predicted by additivity at environmental levels of exposure. Underlining indicates a test group in the hypothetical bioassay.

TABLE 2

RELATIVE AUGMENTED RISK (R*) OF JOINT EXPOSURE COMPARED WITH SUM OF RISK ASSOCIATED WITH SINGLE EXPOSURES

$$\Delta = \frac{\beta_{01}x_1}{\alpha_0} = \frac{\beta_{k-1}x_2}{\alpha_{k-1}}$$

and

$$R^* = \int_0^t \left\{h[x_1(v),x_2(v)] - h(0,0)\right\} dv \div \int_0^t \left\{h[x_1(v),0] + h[0,x_2(v)] - 2h(0,0)\right\} dv$$

	Time Interval					R*		
	0 to t/2		t/2 to t					
No.	$x_1(t)$	$x_2(t)$	$x_1(t)$	$x_2(t)$	Functional Form of R*	k=2	k=5	k=∞
1	0	0	x_1	x_2	$1 + \Delta/2^k$	$1 + \frac{\Delta}{4}$	$1 + \frac{\Delta}{32}$	1
2	0	x_2	x_1	0	1	1	1	1
3	x_1	0	0	x_2	$1 + \Delta\left(\frac{2^{k-1}-1}{2^k-1}\right)$	$1 + \frac{\Delta}{3}$	$1 + \frac{15\Delta}{31}$	$1 + \frac{\Delta}{2}$
4	x_1	x_2	0	0	$1 + \Delta/2^k$	$1 + \frac{\Delta}{4}$	$1 + \frac{\Delta}{32}$	1

TABLE 3

RESPIRATORY CANCER DATA FROM DOLL AND PETO (1978)

	Average Exposure (Cigarettes per Day)--x_1								
Median Age	0.0	2.7	6.6	11.3	16.0	20.4	25.4	30.2	38.0
42.5 years old									
No. of cancers observed	0.0	0.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0
No. of person-years observed	17,846.5	1,216.0	2,041.5	3,795.5	4,824.0	7,046.0	2,523.0	1,715.5	892.5
47.5 years old									
No. of cancers observed	0.0	0.0	0.0	1.0	1.0	1.0	2.0	2.0	0.0
No. of person-years observed	15,832.5	1,000.5	1,745.0	3,205.0	3,995.0	6,460.5	2,565.5	2,123.0	1,150.0
52.5 years old									
No. of cancers observed	1.0	0.0	0.0	2.0	4.0	6.0	3.0	3.0	3.0
No. of person-years observed	12,226.0	853.5	1,562.5	2,727.0	3,278.5	5,583.0	2,620.0	2,226.5	1,281.0
57.5 years old									
No. of cancers observed	2.0	1.0	0.0	1.0	0.0	8.0	5.0	6.0	4.0
No. of person-years observed	8,905.5	625.0	1,355.0	2,288.0	2,466.5	4,357.5	2,108.5	1,923.0	1,063.0
62.5 years old									
No. of cancers observed	0.0	1.0	1.0	1.0	2.0	13.0	4.0	11.0	7.0
No. of person-years observed	6,248.0	509.5	1,068.0	1,714.0	1,829.5	2,863.5	1,508.5	1,362.0	826.0
67.5 years old									
No. of cancers observed	0.0	0.0	1.0	2.0	2.0	12.0	5.0	9.0	9.0
No. of person-years observed	4,351.0	392.5	843.5	1,214.0	1,237.0	1,930.0	974.5	763.5	515.0
72.5 years old									
No. of cancers observed	1.0	1.0	2.0	4.0	4.0	10.0	7.0	2.0	5.0
No. of person-years observed	2,723.5	242.0	696.5	862.0	683.5	1,055.0	527.0	317.5	233.0
77.5 years old									
No. of cancers observed	2.0	0.0	0.0	4.0	5.0	7.0	4.0	2.0	2.0
No. of person-years observed	1,772.0	208.5	517.5	547.0	370.5	512.0	209.5	130.0	88.5

NOTE: No. of person-years observed refers to the total number of person-years observed in that age group at that exposure level.

TABLE 4

RESPIRATORY CANCER AND CIGARETTE SMOKING DATA: MAXIMUM LIKELIHOOD ESTIMATES OF PARAMETERS IN THE MULTISTAGE MODEL

Coefficient	Maximum Likelihood Estimate
A	$0.283404971489 \cdot 10^{-10}$
B_{01}	0.575320316865
$B_{k-1,1}$	0.310436883121
Lag time (w)	13.6420002494

NOTE: This is a five-stage model; stages 1 and 5 are affected. The age at the beginning of exposure is 19.2 years.

TABLE 5

RESPIRATORY CANCER AND CIGARETTE SMOKING DATA: GOODNESS OF FIT OF MODEL TO OBSERVED DATA

	Average Exposure (Cigarettes per Day)--x_1								
Median Age	0.0	2.7	6.6	11.3	16.0	20.4	25.4	30.2	38.0
42.5 years old									
No. of cancers observed	0.0	0.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0
No. of cancers predicted	0.35677	0.044789	0.12817	0.36372	0.63110	1.1651	0.52138	0.42688	0.20605
47.5 years old									
No. of cancers observed	0.0	0.0	0.0	1.0	1.0	1.0	2.0	2.0	0.0
No. of cancers predicted	0.50966	0.072232	0.22458	0.66898	1.1749	2.4918	1.2848	1.3210	0.96899
52.5 years old									
No. of cancers observed	1.0	0.0	0.0	2.0	4.0	6.0	3.0	3.0	3.0
No. of cancers predicted	0.70990	0.11169	0.30437	1.1325	2.0262	4.6780	2.9439	3.1913	2.5759
57.5 years old									
No. of cancers observed	2.0	1.0	0.0	1.0	0.0	8.0	5.0	6.0	4.0
No. of cancers predicted	0.93282	0.13916	0.59754	1.7848	2.9627	7.2796	4.8327	5.7248	4.5421
62.5 years old									
No. of cancers observed	0.0	1.0	1.0	1.0	2.0	13.0	4.0	11.0	7.0
No. of cancers predicted	1.2090	0.18314	0.79696	2.3492	3.9663	8.7946	6.4591	7.6655	6.7739
67.5 years old									
No. of cancers observed	0.0	0.0	1.0	3.0	2.0	12.0	5.0	9.0	9.0
No. of cancers predicted	1.0375	0.21787	1.0125	3.7598	4.5384	10.167	7.2385	7.5168	7.4629
72.5 years old									
No. of cancers observed	1.0	1.0	2.0	4.0	4.0	10.0	7.0	2.0	5.0
No. of cancers predicted	0.92631	0.19974	1.2875	3.8926	4.0198	8.9962	6.3893	5.1326	5.5837
77.5 years old									
No. of cancers observed	2.0	0.0	0.0	4.0	5.0	7.0	6.0	2.0	2.0
No. of cancers predicted	0.83589	0.24773	1.4189	2.9691	3.3258	6.7363	3.9634	3.2772	3.3246

NOTE: Cells were collapsed so that the predicted value is greater than or equal to 2.5, if possible. Degrees of freedom = 40 - 4 = 36.

TABLE 6

AUGMENTED RISK ASSOCIATED WITH SECOND AGENT FOR VARIOUS SMOKING LEVELS

Cigarettes Smoked per Day on Average	Augmented Risk Associated with Second Agent
0	$1.00 \cdot 10^{-5}$
10	$4.09 \cdot 10^{-5}$
20	$7.18 \cdot 10^{-5}$
40	$13.36 \cdot 10^{-5}$

DISCUSSION

Paul I. Feder, Battelle Columbus Division

I enjoyed reading the Thorslund and Charnley paper. It is well written and presents good methodology and useful applications.

The main theme of the paper is the description and estimation of health risks associated with low dose environmental exposures to multiple agent mixtures. Determination of the presence, absence, nature, and extent of interactions among mixture components at low environmental exposure levels is of considerable importance. A key idea of the paper is that the presence or absence of empirically determined, *high dose* interactions observed in laboratory bioassays is *irrelevant* to inferring the presence or absence of interactions among mixture components at low environmental levels of exposure.

In order to make definitive statements about the presence and nature of interactions at low, environmental exposure levels, it is necessary to understand the biological and chemical mechanisms by which the mixture components interact with one another and with the body. There may be chemical interactions among mixture components; differential behavior among components with respect to environmental transformation and fate; saturation of various internal enzymatic processes of metabolism, detoxification, or genetic repair by some mixture components, thereby altering the effects of others. Certain mixture components may modify the pharmacokinetic characteristics of other components, thereby altering their concentrations at the site of action. Individual mixture components may not be carcinogenic, just combinations as with initiators and promoters. Any mechanistic information concerning the modes of action of the mixture components and their interrelations should be incorporated into the dose-response models that extrapolate the observed high dose laboratory effects to predict health effects at the low environmental exposure levels. Thorslund and Charnley assume away many of these mechanistic and pharmacokinetic considerations when they state "...For the mathematical model of the carcinogenic response...it will be assumed that the best available surrogate measure of dose at the site of action is used as the independent variable...". This is easier to assume than to verify. In all fairness though, the biological mechanisms of action are often not very well understood.

Thorslund and Charnley generalize the multistage model to account for multiple agents and variable exposure. Their models are a class of empirical dose response models that predict health effects due to joint exposure, based on individual component data. The models are motivated by the mechanistic considerations underlying the multistage model and provide a plausible explanation of many high dose interactions that are observed in laboratory data. In the absence of specific information about the nature and extent of the biological mechanisms and interactions, this class of models offers a workmanlike approach to describing the low dose behavior of mixtures and the low dose interactions that are operative, among the mixture components. It provides an empirical extension of component additivity to incorporate linear by linear interaction terms into the predictions.

Thorslund and Charnley state "... At high doses the multiplicative exposure effect term can dominate...and the joint effect can be much greater than the sum of the individual effects. However, if both exposures are reduced by several orders of magnitude, the joint effect would be, to a very close approximation, equal to the sum of the individual effects...the multistage model predicts additivity at environmental exposure levels for almost all situations that would be routinely encountered".

While in principle the Thorslund and Charnley model implies low dose component additivity, the viewpoint above is somewhat of an overstatement. It discounts pharmacokinetic interactions such as saturation of elimination or repair processes and it ignores the question of what constitutes a "low" dose. Several examples will be presented below in which the Thorslund and Charnley model is predictive of joint toxicity of a two component mixture at environmental levels of exposure, but yet where component additivity does not hold. Thus, the Thorslund and Charnley model is *not* synonymous with component additivity of risks at low, environmental exposure levels.

Implications of the Thorslund and Charnley Model

The simplest example of the Thorslund and Charnley model corresponds to the case of a two component mixture and two stages. Let U, and V denote the concentrations of components 1 and 2; assume that each component affects a different stage. Equation (13) expresses the risk of a tumor for this special case as

$$P(U,V) = 1 - \exp[-A(1+BU)(1+CV)] \qquad (13)$$

At low environmental exposure levels, P(U,V) can be approximated as

$$\begin{aligned} P(U,V) &\cong A + ABU + ACV + ABUCV \\ &\cong P00 + P10 + P01 + P11. \end{aligned}$$

In this expression P00 represents the background risk, P10 and P01 represent the additional risks due to each component separately, and P11 represents a linear by linear interaction term between components 1 and 2. When P11 is small relative to P10 and P01, the component additional risks are essentially additive. Thus, to determine when component additivity is a reasonable assumption it is necessary to determine conditions under which P11 is small relative to P10, P01. The expressions for P00,P10,P01,P11, imply that

$$\begin{aligned} P11 &= P10P01/P00 = (P10/P00)P01 \\ &= (P01/P00)P10. \end{aligned}$$

Thus P11 is small relative to P10,P01 if

$$P10/P00 \ll 1 \text{ and } P01/P00 \ll 1.$$

Define a *relative risk* as the ratio of the absolute risk to the background risk. That is, let

$R(U,V) = P(U,V)/P00$, $R10 = P10/P00$, $R01 = P0/P00$, and $R11 = P11/P00$. Then $R(U,V) = 1 + R10 + R01 + R10R01$.

The product term is small if $R11 \equiv R10R01 \ll 1$.

Therefore, what constitutes a "low" dose in the Thorslund and Charnley model depends on the level of background risk. To have additive componentwise risks, the additional risks associated with each component must be small compared to the background risk, or equivalently the relative risks must be small compared to 1. If the additional risks for each component are large relative to background, the product term will dominate; the component effects will appear to interact.

Reif (1984) presents a number of epidemiological examples that show the relationship between joint effects and individual component effects at environmental levels of exposure. We illustrate the predictiveness or lack thereof of the Thorslund and Charnley model for these examples.

1. Lung cancer associated with smoking (component 1) and uranium mining (component 2).

$$\begin{aligned} P00 &= .57 \times 10^{-4} & & \\ P00 + P10 &= 5.87 \times 10^{-4} & \quad P10/P00 &= 9.30 \\ P00 + P01 &= 2.27 \times 10^{-4} & \quad P01/P00 &= 2.98. \end{aligned}$$

The additional component risks are large compared to background. The joint risk estimated by the Thorslund and Charnley model is

$$\begin{aligned} 10^4\, P\,(U,V) &= .57 + (5.87 - .57) + (2.27 - .57) \\ &\quad + (5.87 - .57)(2.27 - .57)/.57 \\ &= .57 + 5.3 + 1.7 + 15.81 = 23.38. \end{aligned}$$

The observed value is 22.7. In this example the component additional risks are large relative to background, the product term dominates, and the components appear to be interactive. The Thorslund and Charnley model is predictive at the environmental level of exposure but componentwise additivity does not hold there.

2. Lung cancer associated with smoking (component 1) and asbestos work (component 2).

$$\begin{aligned} P00 &= 1.13 \times 10^{-4} & & \\ P00 + P10 &= 12.3 \times 10^{-4} & \quad P10/P00 &= 9.88 \\ P00 + P01 &= 5.84 \times 10^{-4} & \quad P01/P00 &= 4.17. \end{aligned}$$

The additional component risks are large relative to background. The joint risk estimated by the Thorslund and Charnley model is

$$\begin{aligned} 10^4 P(U,V) &= 1.13 + (12.3 - 1.13) + (5.84 - 1.13) \\ &\quad + (12.3 - 1.13)(5.84 - 1.13)/1.13 \\ &= 1.13 + 11.17 + 4.71 + 46.56 \\ &= 63.57. \end{aligned}$$

The observed value is 60.2. In this example, as in the first, the component additional risks are large relative to background, the product term dominates, and the components appear to be interactive. The Thorslund and Charnley model is predictive at the environmental level of exposure but componentwise additivity does not hold there.

3. Lung cancer associated with smoking (component 1) and asbestos mining (component 2)

$$\begin{aligned} R00 &= 1 \\ R00 + R10 &= 12 \\ R00 + Ro1 &= 1.6. \end{aligned}$$

The additional relative risk for smoking is large whereas that for asbestos mining is not.

$$R(U,V) = 1 + 11 + 0.6 + 6.6 = 19.2.$$

The observed value is 19.0. In this example the component 1 additional relative risk is large, the product term is large relative to the additional effect for component 2, and the components appear to be interactive. The Thorslund and Charnley model is predictive at the environmental level of exposure but componentwise additivity does not hold there.

4. Abnormal sputum cytology associated with smoking (component 1) and uranium mining (component 2)

$$\begin{aligned} P00 &= .04 & & \\ P00 + P10 &= .11 & \quad P10/P11 &= 1.75 \\ P00 + P01 &= .08 & \quad P01/P11 &= 1.0. \\ P(U,V) &= .04 + .07 + .04 + (.07)(.04)/.04 & & \\ &= .22. & & \end{aligned}$$

The observed value is .22. In this example the component additional risks are comparable or moderately large relative to background. The Thorslund and Charnley model is predictive at the environmental level of exposure but componentwise additivity does not hold there.

5. Oral cancer associated with smoking (component 1) and alcohol use (component 2)

$$\begin{aligned} R00 &= 1 \\ R00 + R10 &= 1.53 \\ R00 + R01 &= 1.23. \end{aligned}$$

The additional componentwise relative risks are small relative to background.

$$R(U,V) = 1 + .53 + .23 + (.53)(.23) = 1.88.$$

The observed value is 5.71. In this example the Thorslund and Charnley model predicts essentially componentwise additivity; it is not predictive at the environmental level of exposure.

6. Renal cancer associated with smoking (component 1) and exposure to cadmium (component 2)

$$\begin{aligned} R00 &= 1 \\ R00 + R10 &= 1 \\ R00 = R01 &= .8. \end{aligned}$$

The additional component relative risks are essentially zero. Thus, $R(U,V)$ is at most 1. The observed joint relative risk is 4.4. In this example the Thorslund and Charnley model predicts componentwise additivity; it is not predictive at the environmental level of exposure.

The performance of the Thorslund and Charnley model with Reif's examples has a number of implications.

1. The model predicts some observed component interactions at environmental levels.
2. The model does not predict all observed component interactions at environmental levels.
3. Environmental exposure levels in a number of the examples were sufficiently high for the product term in the model's expression for risk to dominate. Thus, the Thorslund and Charnley model is not synonymous with componentwise additivity of risks at environmental exposure levels.
4. What constitutes "low" levels of exposure and "high" levels of exposure for the purposes of the model is based on risk levels relative to background. Exposure levels that might be quite low on an absolute basis could still be "high" with respect to componentwise additivity in the Thorslund and Charnley model.
5. Irrespective of whether or not the model predicts component additivity, inferences concerning the joint effects of multiple components can be based on individual component data alone. Component data are the most readily available for risk assessment purposes.

Conclusions

The USEPA Guidelines for the Health Risk Assessment of Chemical Mixtures (1985), page 12 state "...When little or no quantitative information is available on the potential interactions among the components, additive models are recommended for systemic toxicants...". This paper carries the above recommendation a step further; the model accounts for linear by linear interactions empirically, based on component data. This provides a good, empirical modeling approach in the absence of specialized mechanistic information. The model does not always predict componentwise additivity at low, environmental levels of exposure. It predicts some, but not all, observed environmental interactions among mixture components.

What level of subdivision of the mixture into components should be used when carrying out the risk calculations? If the composite, tested as a whole, is not carcinogenic at the laboratory dose levels, can testing be stopped without considering componentwise tests? I believe that the answer is no! The USEPA Mixtures Guidelines (1985), page 11 state "...Even if a risk assessment can be made using data on the mixture of concern or a reasonably similar mixture, it may be desirable to conduct a risk assessment based on toxicity data on the components in the mixture...in a chronic (high dose) study of such a mixture (containing carcinogens and toxicants), the presence of the (acute) toxicant could mask the activity of the carcinogen...the toxicant could induce mortality (at high doses) so that at the maximum tolerated dose of the mixture, no carcinogenic effect could be observed...". However, at low, environmental levels of exposure the acute toxicant might have no effect and so the carcinogenic component might be active. "...The mixture approach should be modified to allow the risk assessor to evaluate the potential for masking, of one effect by another, on a case-by-case basis".

Thus, a sensible empirical approach to carrying out risk assessments on mixtures in the absence of specific mechanistic information concerning componentwise interactions, would be to carry out dose response estimation and risk calculations based on componentwise testing at a number of different levels of decomposition of the mixture, ranging from the entire composite to very homogeneous constituents. At each level of decomposition the componentwise risks would be combined based on the Thorslund and Charnley model to obtain composite risk estimates. Large discrepancies in the composite risk estimates at differing levels of decomposition would indicate the presence of synergistic or antagonistic component interactions.

In conclusion, I found this paper to be very interesting, thought provoking, and well written. It raises as many or more questions about methodology for risk assessment on mixtures as it resolves.

References

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ASSESSMENT OF INTERACTION IN LONG-TERM EXPERIMENTS

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1. INTRODUCTION

This paper will address the problem of assessing interaction between carcinogens or toxic substances in long-term factorially designed animal experiments. The general context is that of long-term screening tests for carcinogens, for which the analysis is based on Cox regression methods, see, for instance, Peto et al. (1980). The designs considered are 2 x 2 and 2 x 2 x 2. The method easily generalizes to other factorial designs. The problem came to the author's attention via a request from Dr. C.J. Shellabarger of Brookhaven National Laboratory who had completed a 2 x 2 x 2 experiment to examine interactions between radiation and chemical carcinogens in the induction of mammary tumors in rats, and was not sure how to analyze his data. He had previously conducted 2 x 2 experiments and to add a third treatment seemed a natural next step.

In the statistical and epidemiological literature, there has been a lot of discussion in recent years about what is meant by interaction. Distinctions have been made between statistical and biological interaction, and interaction in the public health sense. As statisticians are well aware, presence of interaction in a linear model depends on the scale of measurement being used. For instance, for a two-way layout with one observation per cell, interaction can be "got rid of" by a suitable power transformation of the data. To reduce confusion, in an area which is complicated enough, it is important for the statistician to define what is meant by interaction at the outset of a study, and what is meant by "synergism" and "antagonism," since these terms do not mean the same things to all scientists.

In this paper, the kind of interaction considered, between agents A and B, is that which occurs if the effect of A and B taken together is unexpectedly larger or smaller than that of the sum of the effects of A and B taken separately. Synergism is said to occur if the joint effect is larger than expected, and, conversely, antagonism occurs if the joint effect is smaller than expected. The situation in which only A produces the effect of interest, but the presence of B modifies the effect of A, is not considered. A and B are presumed to have the same site of action.

The underlying model for no interaction considered here is Finney's definition of simple independent action of different agents and the background (1971). This is equivalent to Hewlett and Plackett's model of "dissimilar noninteractive action."

For long-term experiments, under the proportional hazards assumption, the model results in a linear relationship between the hazard functions rather than the multiplicative one commonly assumed for interaction (see Wahrendorf et al., 1981). Other researchers have looked at additive as well as multiplicative models for no interaction, for instance, Thomas (1981) in the context of general relative risk models, and Prentice et al. (1983) for the analysis of an extensive cohort study.

The work for this paper was done with the assistance of Dr. Kent Bailey, of the National Heart, Lung and Blood Institute, and is essentially a continuation of that of Wahrendorf et al. The contribution of Korn and Liu (1983) who took a non-parametric approach, i.e., without making the proportional hazards assumption, will be briefly mentioned.

2. 2 x 2 FACTORIAL EXPERIMENTS

The hypothesis to be tested is that of independence of action, i.e., of tumor inducing potential, between carcinogens given in combination. Suppose, in a 2 x 2 experiment with treatments A and B, that n_{00} animals receive no treatment and n_{10}, n_{01}, n_{11} animals receive, respectively, doses d_A of A, d_B of B and $(d_A + d_B)$. The animals are observed throughout the experiment for occurrence of tumors of interest; times of tumor appearance or of deaths from unrelated causes are noted. Let $q_{ij}(t)$, $i,j=0,1$, be the probability that an animal in the group (i,j) remains tumor-free up to time t. Let $Q_{ij} = q_{ij}(T)$ where T is any time after the last event. Let m_{ij} be the number of animals with tumors in group (i,j) at time T. Finney's hypothesis of simple independent action of A and B is:

$$H_0:\ Q_{11}Q_{00} = Q_{10}Q_{01} \quad .$$

Synergism corresponds to the left hand side being much less than the right hand side of this expression; conversely for antagonism.

Various methods have been proposed for testing this hypothesis, see Wahrendorf et al., Korn and Liu, or Hogan et al. (1978). Perhaps the simplest conceptually is the likelihood ratio test. The likelihood is proportional to:

$$\Pi_{ij}(1 - Q_{ij})^{m_{ij}}\ Q_{ij}^{\ n_{ij}-m_{ij}} \quad .$$

The log-likelihood is first maximized with $\{Q_{ij}\}$ as four independent parameters, i.e., $\hat{Q}_{ij} = (n_{ij}-m_{ij})/n_{ij}$, and then with the $\{Q_{ij}\}$ constrained by the null hypothesis.

Taking time into account, Finney's hypothesis of independent action of A and B becomes:

$$H_0:\ \log\left([q_{11}(t)q_{00}(t)]/[q_{10}(t)q_{01}(t)]\right) = 0$$

for all t.

Let $\lambda_{ij}(t) = -(d/dt)\ \{\log(q_{ij}(t))\}$ denote the hazard function for the ocurrence of a tumor for an animal in the group (i,j); then H_0 becomes:

$$\int \{\lambda_{11}(t) + \lambda_{00}(t) - \lambda_{10}(t) - \lambda_{01}(t)\}\ dt = 0$$

for all t, or equivalently,

$$\lambda_{11}(t) + \lambda_{00}(t) - \lambda_{10}(t) - \lambda_{01}(t) = 0 \qquad (1)$$

for all t.

Synergism corresponds to the expression on the left of (1) being greater than zero and, conversely, antagonism corresponds to the expression being less than zero.

If the proportional hazards assumption is made, namely that $\lambda_{ij}(t)$ may be expressed as $\lambda_{00}(t)f_{ij}$ where f_{ij} does not depend on t, then the null hypothesis becomes, without loss of generality:

$$H_0: \; 1 + f_{11} - f_{10} - f_{01} = 0 \, ,$$

the arbitrary scale factor having been absorbed into the arbitrary function $\lambda_{00}(t)$. The alternative hypothesis allows for all three parameters to be free. Let $f = (f_{00}, f_{10}, f_{01}, f_{11})'$. For testing H_0 it is convenient to express f in terms of parameters $\{\beta\}$. Let $T_M(\beta) = (1, e^{\beta_1}, \ldots, e^{\beta_M})'$ for arbitrary $M >= 1$ and let W be any 4x3 matrix of constants with columns orthogonal to the interaction contrast vector $(1,-1,-1,1)' = v_{AB}$, say. There are many choices for W; a natural choice is to define W as the first three columns of the design matrix

$$\begin{pmatrix} 1 & -1 \\ 1 & 1 \end{pmatrix} \otimes \begin{pmatrix} 1 & -1 \\ 1 & 1 \end{pmatrix} \, .$$

Under H_0, f is modelled as $f = WT_2(\beta)$, and under the alternative hypothesis, $f = T_3(\beta)$. The null hypothesis H_0 is tested by the likelihood ratio statistic, $X = 2(L_S - L_C)$, where L_S, L_C are, respectively, the maximized log-likelihood functions under the saturated and constrained models; X has approximately χ_1^2 distribution under the null hypothesis. Technical details for the likelihood maximization are described in Machado and Bailey (1985).

3. EXAMPLE 1

The data for this example is from Table 2 of Korn and Liu (1983). Female rats were treated with two chemical carcinogens, labelled NTA and MNNG. The endpoint of interest was death from any cause. All rats alive at the end of the experiment, as well as 4 accidently killed early on, are considered censored observations. The results of the analysis were:

Saturated Model	Constrained Model
$L_S = -291.481$	$L_C = -294.588$
$\hat{\beta} = \begin{pmatrix} 0.61 \\ 2.08 \\ 1.26 \end{pmatrix}$	$\hat{\beta} = \begin{pmatrix} 0.25 \\ 1.56 \end{pmatrix}$
$\hat{f} = \begin{pmatrix} 1 \\ 1.83 \\ 8.00 \\ 3.52 \end{pmatrix}$	$\hat{f} = \begin{pmatrix} 1 \\ 1.29 \\ 4.76 \\ 5.04 \end{pmatrix}$
$v_{AB}'\hat{f} = -5.31$	$v_{AB}'\hat{f} = 0$

The likelihood ratio test, $X = 6.21$ ($\alpha = 0.013$), indicating significant departure from the null hypothesis; the interaction is antagonistic, from the sign of $v_{AB}'\hat{f}$. This result corresponds to those of Korn and Liu.

For comparison, the time-independent likelihood ratio test was $2(L_S - L_C) = 4.32$; this statistic has approximately χ_1^2 distribution, thus $\alpha = 0.039$, again indicating significant interaction.

4. EMPTY CELLS

A problem which may occur is that one or more groups may have no animals with tumors. This leads to an infinite solution for the $\{\beta\}$ in the original parametrization. The model can often be expressed in terms of alternative parameters and maximum likelihood estimates found without the groups which have no animals with tumors. For example, in the 2 x 2 case, if no animal in the control group has a tumor, so that $\lambda_{00}(t) = 0$, this group may be excluded from the analysis and $f_- = (f_{10}, f_{01}, f_{11})$ may be modelled by $W_-T_1(\beta)$ with

$$W_- = \begin{pmatrix} 1 & 0 \\ 0 & 1 \\ 1 & 1 \end{pmatrix}$$

This represents the additive model subject to $f_{00} = 0$. If the parameters are estimated as in Machado and Bailey, the log-likelihood is the log-likelihood for the additive model (1) for all four groups. The saturated model is fitted similarly by omitting the control group and modelling (f_{10}, f_{01}, f_{11}) by $T_2(\beta)$.

5. EXAMPLE 2.

This example was described in Machado and Bailey. The data are from a nine-month study to investigate possible interaction between the known carcinogens diethylstilbestrol (DES) and dimethylbenzanthracene (DMBA) in the induction of mammary adenocarcinomas in female ACI rats (Shellabarger et al., 1980). The results of the test for interaction are:

Saturated Model	Constrained Model
$L_S = -152.46$	$L_C = -156.848$
$\beta = \begin{pmatrix} -0.52 \\ 1.47 \end{pmatrix}$	$\beta = -0.68$
$\hat{f} = \begin{pmatrix} 1 \\ 0.60 \\ 4.33 \end{pmatrix}$	$\hat{f} = \begin{pmatrix} 1 \\ 0.51 \\ 1.51 \end{pmatrix}$
$\hat{f}_{11} - \hat{f}_{10} - \hat{f}_{01} = 2.74$	$\hat{f}_{11} - \hat{f}_{10} - \hat{f}_{01} = 0$

The test statistic $X = 8.77$ ($\alpha = 0.004$), indicating very significant departure from the null hypothesis. Since the contrast, $\hat{f}_{11} - \hat{f}_{10} - \hat{f}_{01}$, is greater than 0, the interaction is synergistic.

The likelihood ratio test from the time-independent test is 6.61 ($\alpha = 0.01$), again indicating significant interaction.

6. PROPORTIONAL HAZARDS ASSUMPTION.

The proportional hazards assumption should be checked since gross departures from proportionality may well affect the behavior of the interaction tests in an adverse way. Kalbfleisch and Prentice (1980, ch. 4) recommend using "log-minus-log" plots: plots for all the treatment groups of $\log(-\log(q(t))$ versus $\log(t)$, where $q(t)$ is any estimate of $q_{ij}(t)$, will show constant separation over time if there is proportionality. For small to moderate sized samples, it may be difficult to discern from the plots whether there actually is constant separation over time. In this case the uniformity of the fit of the saturated and constrained models and of the behavior of the interaction statistics over time may be checked by estimating sets of parameters for different partitions of the time axis. Most likely, divisions into early versus late, or early, middle and late time periods will be sufficient.

Suppose T is any time beyond the time to the last event and let a time t be chosen so that the time axis is partitioned into $(0,t]$, $(t,T]$. Let $L_S(1)$, $L_S(2)$ be the maximized log-likelihoods under the saturated model, respectively, for the time periods $(0,t]$ (events after t considered censored) and $(t,T]$ (individuals with either events or censoring times before t excluded from the analysis); let $L_C(1)$, $L_C(2)$ be similarly defined maximized log-likelihoods for the constrained model. Then a test for the uniformity of the saturated model over time is $2(L_S(1) + L_S(2) - L_S)$ which has approximate chi-square distribution with degrees of freedom 3 for the 2 x 2 case. A test for the uniformity of the fit of the constrained model over time is $2(L_C(1) + L_C(2) - L_C)$ which has approximate chi-square distribution with degrees of freedom the number of parameters in the model, e.g., 2 in the 2 x 2 case. If there appears to be no lack of uniformity of fit of the saturated or constrained models and no evidence of any interaction, then one would be comfortable in accepting the null hypothesis. If there seems to be evidence of interaction and the interaction is of the same type in each time period, and also if there is uniformity of fit of the saturated model, then the overall test of interaction under the proportional hazards assumption can be used. If there is evidence of nonproportionality, the two time periods could be considered separately with respect to the presence or absence of interaction. It is worth checking the consistency of the conclusions of such an analysis when different values of t are chosen.

If the proportional hazards assumption does not appear to hold, with different partitions of the time axis, then the non-parametric methods of Korn and Liu (1983) may be more appropriate.

7. EXAMPLES

Figure 1 shows the "log-minus-log" plot for the data of Korn and Liu discussed in Example 1. There is no reason to suspect departure from proportionality of the hazard function.

Figure 2 shows the "log-minus-log" plot for the data of Example 2. The plot indicates some departure from proportionality since the curve for the group receiving both DES and DMBA seems steeper than those of the groups receiving a single treatment. The time axis was partitioned into two periods: (0,136) and (137,266), day 136 being approximately the half-way point in time and in numbers of events. The maximized log-likelihoods and estimated parameters for the saturated model were, for the early time period: $L_S(1) = -80.63$, $L_C(1) = -83.36$, $(\hat{\beta}_1, \hat{\beta}_2) = (-1.27, 1.29)$ and for the later time period: $L_S(2) = -71.00$, $L_C(2) = -72.78$, $(\hat{\beta}_1, \hat{\beta}_2) = (-0.02, 1.67)$. The test for homogeneity of fit of the saturated model is $\chi_1^2 = 1.65 (\alpha > 0.10)$ indicating no strong evidence for lack of uniformity; the estimated β coefficients are not very similar but are far from significantly different. The χ_1^2 tests for interaction for the early and late time periods are 5.45 ($\alpha = 0.020$) and 3.56 ($\alpha = 0.059$), respectively; moreover, the interaction contrasts are 2.35 and 3.32 and thus there is significant synergism for both time periods. Similar results were obtained for various t between 129 and 190. Thus it appears that there was a synergistic interaction between DES and DMBA in this experiment.

8. KORN AND LIU'S Z STATISTIC

Korn and Liu proposed a statistic for continuous time data which does not rely on the proportional hazards assumption. They made the reasonable suggestion that the model of independent action with no further assumptions is a good starting place for an analysis.

Restricting attention to the 2 x 2 case, suppose events occur at time $t_1, t_2, \ldots$, and suppose that there are no tied events. Let

$$Z_k = (-1)^{i+j+1} n_{++}(t_k)/n_{ij}(t_k)$$

where $n_{ij}(t_k)$ is the number of animals in group (i,j) exposed at time $t_k - 0$, $n_{++}(t_k) = \Sigma_{ij} n_{ij}(t_k)$, and the failure occurred in group (i,j). The statistic for testing Finney's hypothesis of independent action is:

$$Z = \frac{\Sigma Z_k}{(\Sigma Z_k^2)^{1/2}}$$

which has approximately N(0,1) distribution. The terms in the numerator, $\{Z_k\}$, have conditional expectation zero under the null model, and in this, are unique up to a multiplicative factor.

If there are ties in the data, Korn and Liu suggest breaking them at random. Note that with the test discussed in this paper, it is possible to use the general maximum likelihood solution to the proportional hazards model, and thus ties do not pose a problem (see Machado and Bailey).

Korn and Liu's statistic was calculated for the data of Examples 1 and 2:

Example 1.

$Z = 2.45$ ($\alpha = 0.014$) compared with $\sqrt{X} = \sqrt{6.21} = 2.49$ ($\alpha = 0.013$). Note that in their paper, they obtained $Z = 2.48$, a minor difference, but due to arbitrariness in dealing with ties. For this example, the likelihood ratio test and Z are very close.

Example 2.

With the data ordered by treatment group: Controls, DES, DMBA, DES plus DMBA, $Z = -1.87$ ($\alpha = .06$); with the data ordered in reverse, i.e., DES plus DMBA, DMBA, DES, Controls, $Z = -1.645$ ($\alpha = 0.10$). There are a lot of ties in the data, which give rise to the difference between these two values of Z. These values are rather different from $\sqrt{8.77} = 2.96$ ($\alpha = 0.004$) from the time-dependent likelihood ratio test, and $\sqrt{6.61} = 2.57$ ($\alpha = 0.01$) of the time-independent test. It is difficult to see why the Z values should indicate less evidence of the synergism between DES and DMBA.

Once a consistent approach to dealing with ties is found, the Z statistic should prove useful, since it is reasonably simple to compute, especially for situations in which one feels uneasy about assuming proportionality of hazards.

9. 2*2*2 FACTORIAL EXPERIMENTS

Let the three treatments of interest be A, B and C and suppose that there are n_{ijk} animals in the group (i,j,k) receiving a total treatment dose of $(id_A + jd_B + kd_C)$, for $i,j,k=0,1$. Let $q_{ijk}(t)$, $\lambda_{ijk}(t)$ be defined in an analogous way to $q_{ij}(t)$, $\lambda_{ij}(t)$ of Section 2. Further, under the proportional hazards assumption, let $\lambda_{ijk}(t) = \lambda_{000}(t)f_{ijk}$, where f_{ijk} is independent of t. Let $q(t)$ be the vector of the $\{q_{ijk}(t)\}$ with the subscripts in the order (000,100,010,110,001,101,011,111) and let f be the vector of $\{f_{ijk}\}$ with the subscripts in the same order. Let the columns of the design matrix

$$\begin{pmatrix} 1 & -1 \\ 1 & 1 \end{pmatrix} \otimes \begin{pmatrix} 1 & -1 \\ 1 & 1 \end{pmatrix} \otimes \begin{pmatrix} 1 & -1 \\ 1 & 1 \end{pmatrix}$$

be labelled in order, v_0, v_A, v_B, v_{AB}, v_C, v_{AC}, v_{BC}, v_{ABC}. Then the null hypothesis of independence of action of the three treatments has four parts, corresponding, respectively to the interactions between A and B, A and C, B and C, and A, B and C.:

$$H_0: \text{(i)}\ v_{AB}'\log(q) = 0\ ,\ \text{(ii)}\ v_{AC}'\log(q) = 0,$$
$$\text{(iii)}\ v_{BC}'\log(q) = 0,\ \ \text{(iv)}\ v_{ABC}'\log(q) = 0$$

Under the proportional hazards assumption, as in the previous section, H_0 becomes:

$$\text{(i)}\ v_{AB}'f = 0 \qquad \text{(ii)}\ v_{AC}'f = 0$$
$$\text{(iii)}\ v_{BC}'f = 0 \qquad \text{(iv)}\ v_{ABC}'f = 0$$

A joint test of the four parts of H_0 is made by comparing the constrained model $f = WT_3(\beta)$, where W is an 8 x 4 matrix of constants with columns orthogonal to v_{AB}, v_{AB}, v_{BC} and v_{ABC}, with the saturated model, $f = WT_7(\beta)$; the resulting likelihood ratio statistic has approximately χ_4^2 distribution. One choice for W is to take as its columns v_0, v_A, v_B, v_C. Sequential tests for single interactions are made by modelling the specific constraints by suitable choice of W. For example, to test for the three-way interaction, model f by $W_1T_6(\beta)$, where W_1 is 8 x 7, orthogonal to v_{ABC}, and make a one degree of freedom comparison with the saturated model. Further, to then test for the AB interaction, model f by $W_2T_5(\beta)$, where W_2 is 8 x 6, orthogonal to v_{AB} and v_{ABC}, and compare with the model $W_1T_6(\beta)$. Leaving out one constraint at a time leads to a series of one-degree of freedom comparisons in the usual way.

10. EXAMPLE 3

The data for this 2 x 2 x 2 example are from a one-year experiment to assess possible interaction between DMBA, procarbazine (PCZ) and x-irradiation (X-ray) in the induction of mammary adenocarcinomas or fibroadenomas in female Sprague-Dawley rats. The experiment was conducted by Dr. C.J. Shellabarger and colleagues at Brookhaven National Laboratory who kindly made the data available. The rats were treated at about 3 months of age and examined weekly for the appearance of mammary tumors. Summary information on numbers of rats with one or more tumors is in Table 1.

Table 1. Summary information on numbers of female rats treated with DMBA, PCZ and/or X-ray which developed mammary tumors.

Treatment group	Number exposed	Number with at least one tumor
Control	35	2
DMBA	37	14
PCZ	37	8
DMBA & PCZ	37	20
X-ray	37	11
DMBA & X-ray	37	20
PCZ & X-ray	36	14
DMBA, PCZ & X-ray	37	25

The maximized log-likelihoods were: $L_S = 510.981$, $L_C = -511.422$ resulting in test statistic for overall interaction, $\chi_4^2 = 0.822$, which is far from significance. There were no two-way or three-way interactions between DMBA, PCZ and X-ray since the single degree of freedom chi-square tests for individual interactions are all bounded by such a small number. For comparison, the likelihood ratio statistic from the time-independent test is 0.682, also very small. The estimates of the multipliers $\{f_{ijk}\}$ from the saturated and constrained models are very similar:

$$\hat{f} = (1,2.19,1.47,2.66,1.90,2.85,2.18,3.05)' \text{ and}$$
$$\hat{f} = (1,2.42,1.53,2.70,1.96,2.85,2.37,3.04)' .$$

"Log-minus-log" plots showed close to constant separation between all of the curves and thus is there no reason to doubt the proportionality of the hazard functions. Thus the three treatments act independently in the induction of one or more mammary tumors in this species of rat.

For this data, the time to the appearance of second tumors was also recorded. This is a much less understood measure of carcinogenesis, and the analysis is summarized here only for illustration of the method. Table 2 gives the numbers of animals in each treatment group which developed 2 or more mammary tumors.

Table 2. Summary information on numbers of female rats treated with DMBA, PCZ, and/or X-ray which developed 2 or more mammary tumors.

Treatment group	Number exposed	Number with 2 or more tumors
Control	35	0
DMBA	37	5
PCZ	37	1
DMBA & PCZ	37	15
X-ray	37	1
DMBA & X-ray	37	10
PCZ and X-ray	36	3
DMBA, PCZ & X-ray	37	19

The likelihood ratio test for no two-way or three-way interactions was $\chi_4^2 = 10.48$ ($\alpha = 0.035$), indicating the presence of some interaction. The test for no DMBA and X-ray, or PCZ and X-ray, or DMBA, PCZ and X-ray interactions was $\chi_3^2 = 2.77$ ($\alpha > 0.1$), which is far from significance.

The χ_1^2 test for the DMBA and PCZ interaction was 7.71 ($\alpha = 0.006$), which is highly significant. Examination of the statistics showed this to be due to synergism betwen DMBA and PCZ, in the induction of multiple mammary tumors. Although the biological implications of this are not clear, this example shows that the test can identify which pair of agents contributed to the overall departure from the null model.

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FIGURE 1

PLOT OF log(-log(q(t))) vs. log(t)

KORN-LIU DATA

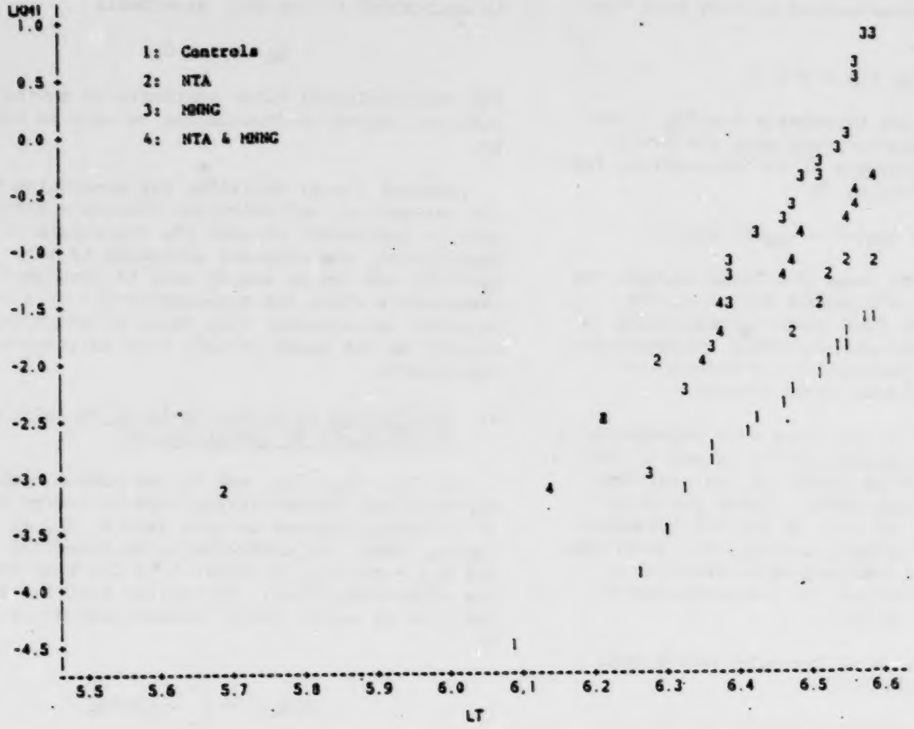
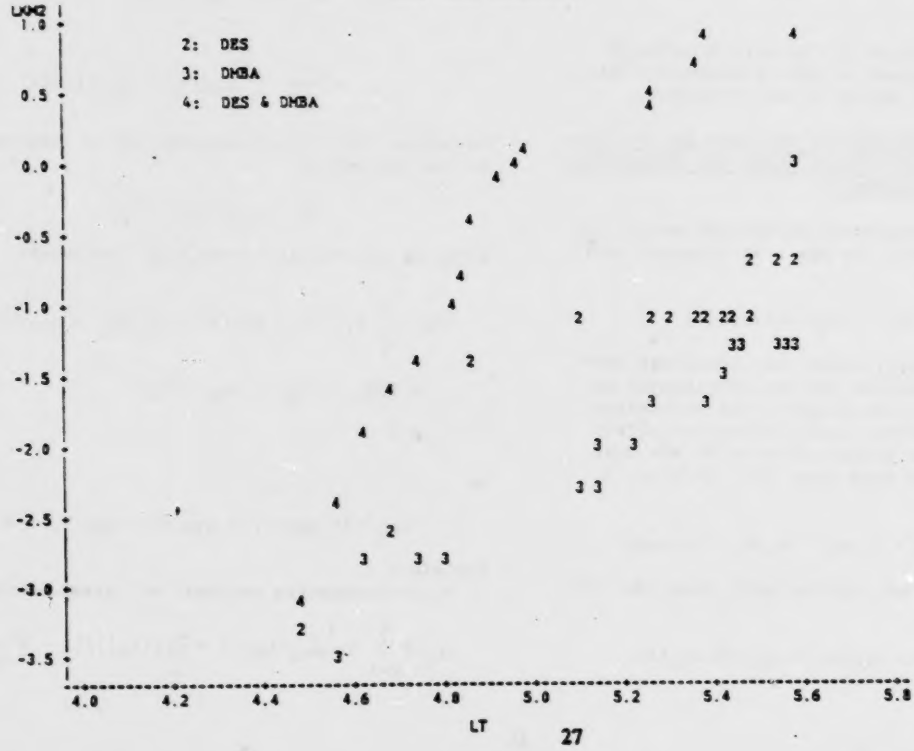


FIGURE 2

PLOT OF log(-log(q(t)) vs. log(t)

DES, DMBA EXPERIMENT



DISCUSSION

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From Machado's presentation we have seen that quantities

$$q_{ijk},\ i,j,k = 0,\ 1$$

play a key role in the hypothesis testing of no interaction when time-to-event data are available. The null hypothesis of no interaction, for the case of a 2x2 design, is

$$H_{01}:\ q_{11}(t)\ q_{00}(t) = q_{10}(t)\ q_{01}(t)$$

for every t. For the case of a 2x2x2 design, the null hypothesis of independent action of the three treatments has four parts corresponding to the three two-way and one three-way interactions.

Given the above background, my discussion will be on the following three issues:

1. Application of the above null hypothesis to test the multiplicative effect of synergism under the theory of the Armitage-Doll multistage model. Under the multistage model, if each of the two (or more) carcinogens affects a single but different stage of the carcinogenesis process, a synergistic effect (in a multiplicative sense) will result.

2. The implication of the alternative null hypothesis of no interaction,

$$H_{02}:\ q_{11}(t) + q_{00}(t) - q_{10}(t) - q_{01}(t) = 0$$

for every t. This alternative hypothesis uses the same information in an additive sense rather than in a multiplicative sense.

3. The implication of the null hypothesis used by Machado to test independent action for a 2x2x2 design of an experiment.

1. Testing the Synergistic (Multiplicative) Effect of Two Carcinogens under the Simple Multistage Carcinogenesis

Under the Armitage-Doll multistage model, the probability of cancer by age t at constant dose rate d has a form

$$P(t,d) = 1 - \exp[-g(t)f(d)]$$

It can be shown that, under the multistage carcinogenesis, if each of the two carcinogens exposed affects only one stage of the multistage process, a synergistic (multiplicative) effect will result if the stages affected by the dose are different. In this case, the function, f, has a form

$$f(d) = b_{00} + b_{10}d_A + b_{01}d_B + b_{11}d_Ad_B$$

It is simple to show algebraically that the null hypothesis

$$H_{01}:\ q_{11}(t)\ q_{00}(t) = q_{10}(t)\ q_{01}(t)$$

is equivalent to the null hypothesis

$$H_0:\ b_{11} = 0.$$

The log-likelihood ratio statistic of chi-square with one degree of freedom can be used to test H_0.

Although P(t,d) satisfies the proportion hazard assumption, and therefore Machado's procedure is applicable to test the hypothesis of no interaction, the proposed procedure is more specific and can be easily used in routine risk assessments where the goodness-of-fit of a dose-response relationship must first be determined, usually on the basis of data from multiple-dose experiments.

2. Alternative Null Hypothesis of No Interaction on the Basis of Latent Period

Let T_{00}, T_{10}, T_{01}, and T_{11} be random variables representing, respectively, time to cancer death of an animal exposed to dose rate 0, d_A, d_B and (d_A+d_B) under the condition of no competing risk. Let $M_{ij} = \min(T_{ij}, L)$ where L is the time when the study terminated. It follows that the expected time to cancer death (latent period) is given by

$$E(M_{ij}) = \int_0^L q_{ij}(t)dt$$

and the life-shortening due to the exposure is

$$R_{ij} = \frac{1}{L} \times E(M_{00} - M_{ij})$$

$$= \frac{1}{L} \int_0^L [q_{00}(t) - q_{ij}(t)]dt$$

Therefore, the null hypothesis of no interaction can be defined as

$$H_0:\ R_{11} = R_{10} + R_{01}$$

which is equivalent to the null hypothesis

$$H_0:\ \int_0^L [q_{11}(t) + q_{00}(t) - q_{10}(t) - q_{01}(t)]dt$$

$$= E[M_{11} + M_{00} - M_{10} - M_{01}]$$

$$= 0$$

or

$$q_{11}(t) + q_{00}(t) - q_{10}(t) - q_{01}(t) = 0$$

for every t.

A non-parametric estimate of latent period is

$$e_{ij} = \sum_{k=1}^{n} t_k[\hat{q}_{ij}(t_{k-1}) - \hat{q}_{ij}(t_k)]/[1 - \hat{q}_{ij}(t_n)]$$

where

$t_1, \ldots, t_n$ are the time to cancer death,
$t_0 = 0$, and
$\hat{q}_{ij}$ = Kaplan-Meier estimate of q_{ij}.

The estimate of standard deviation is given by

$$s_{ij} = \{ \frac{n}{n-1} (\sum_{k=1}^{n} t_k^2 [\hat{q}_{i,j}(t_{k-1}) - \hat{q}_{ij}(t_k)] / [1 - \hat{q}_{ij}(t_n)] - e_{ij}^2) \}^{1/2}$$

The statistic

$$Z = (e_{11} + e_{00} - e_{10} - e_{01}) / (s_{11}^2 + s_{00}^2 + s_{10}^2 + s_{01}^2)^{1/2}$$

is asymptotically distributed as a standard normal under H_0.

3. Implication of the Null Hypothesis used by Machado in a 2x2x2 Design

The null hypothesis used by Machado is

$$H_0: V'f = 0$$

where $V = V_{AB}$, V_{AC}, V_{BC}, or V_{ABC} are column vectors given below:

	V_{AB}	V_{AC}	V_{BC}	V_{ABC}
0	1	1	1	1
A	-1	-1	1	1
B	-1	1	-1	1
AB	1	-1	-1	-1
C	1	-1	-1	1
AC	-1	1	-1	-1
BC	-1	-1	1	-1
ABC	1	1	1	1

Let

$$p_{ijk}, \ i, j, k = 0, 1$$

denote $\log(q_{ijk})$ or f_{ijk} as used by Machado.

The null hypothesis H_0 corresponds to four parts:

$$H_{ABC} = p_{111} - p_{110} - p_{011} - p_{101} + p_{100} + p_{010} + p_{001} - p_{000}$$

$$= (p_{111} - p_{101} - p_{011} + p_{001}) - (p_{110} - p_{100} - p_{010} + p_{000})$$

$$= (E_{AB})_{C=1} + (E_{AB})_{C=0} = 0$$

where $(E_{AB})_{C=1}$ and $(E_{AB})_{C=0}$ are defined by the last equality of the equation and represent, respectively, the effect due to treatment A and B when C is held at level 1 or 0.

$$H_{AB} = (p_{111} - p_{011} - p_{101} + p_{001}) + (p_{110} - p_{100} - p_{010} - p_{000})$$

$$= (E_{AB})_{C=1} + (E_{AB})_{C=0} = 0$$

Similarly,

$$H_{BC} = (E_{BC})_{A=1} + (E_{BC})_{A=0} = 0$$

and

$$H_{AC} = (E_{AC})_{B=1} + (E_{AC})_{B=0} = 0$$

Now, synergistic and antagonistic effects should be as follows:

$$E_{ABC} = E(A,B,C) - E(A) - E(B) - E(C)$$

$$= (p_{111} - p_{000}) - (p_{100} - p_{000}) - (p_{010} - p_{000}) - (p_{001} - p_{000})$$

$$= p_{111} - p_{100} - p_{010} - p_{001} + 2p_{000}$$

This shows that the null hypothesis used by Machado is stronger than the test of no synergetic or antagonistic effects as defined by E_{ABC}. If there is no pairwise interaction [i.e., $E(AB)_{C=0} = 0$, etc.], the null hypothesis H_0 of Machado is equivalent to the null hypothesis of $E_{ABC} = 0$. However, in general, $E_{ABC} = 0$ does not imply H_0 to be true.

Further research is needed in testing the null hypothesis of no synergistic or antagonistic effects on 2x2x2 design, without assuming that the pairwise interactions are zero. It seems intuiively true that if all pairwise interactions are positive, there must be a synergistic effect. On the other hand, if all the pairwise interactions are negative, there must be a synergistic effect.

CONCENTRATION AND RESPONSE ADDITION OF MIXTURES OF TOXICANTS USING LETHALITY, GROWTH AND ORGAN SYSTEM STUDIES

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Dose response relationships are the most single unifying concept to the many branches of pharmacology and toxicology. Quantative methodology to describe dose response relationships began with the work of Trevan (1927) and Gaddum (1933). The theoretical basis of joint toxicant action was first systematically discussed by Bliss (1939). Bliss recognized three types of joint action: (1) Independent joint action - the chemicals act independently and have different modes of action; (2) Similar joint action - the chemicals produce similar but independent effects, one component can be substituted at a constant proportion for the other. Susceptibility to the chemical components are completely correlated; (3) Synergistic (or antagonistic) action - the effectiveness of the mixture cannot be assessed from the individual chemicals. Bliss' approach was modified by Finney (1942) to develop a logical relationship between the mathematical expressions for the different types of joint action.

Plackett and Hewlett (1948, 1952) and Hewlett and Plackett (1952, 1959) proposed a two-way classification scheme of Bliss' model in an effort to provide a less restrictive form. The following diagram is their scheme with four distinct types of action.

	Similar	Dissimilar
Non-Interactive	Simple similar	Independent
Interactive	Complex similar	Dependent

They defined toxicant mixtures as "similar" or "dissimilar" according to whether the toxicants acted upon the same of different biological systems and as "interactive" or "non-interactive" according to whether one toxicant influenced the "biological action" of the other toxicants. "Simple similar" and "independent action" were regarded as special cases in a continuum of biological possibilities and the mathematical models proposed for complex similar and dependent were generalizations of the models proposed for "simple similar" and "independent action" respectively.

Their mathematical models, particularly for the quantal responses to mixtures of "interactive" toxicants, are very complex and require the knowledge of certain parameters which are normally unattainable when evaluating the effects of toxicant mixtures on whole organism performances. However, Hewlett and Plackett's models for "joint action" are useful for elucidating the limitations of and the assumptions required for the special cases of "simple similar" and "independent joint action". The present discussion only considers the special cases of "noninteractive" toxicant mixtures.

The difficulty of understanding complex mixtures and the interactive role that individual toxicants play is not easily elucidated. In 1970 my laboratory began to investigate the toxicity of mixtures of chemicals. In the following decade we utilized the concepts of early investigators such as Bliss (1939), Finney (1942) and Hewlett and Plackett (1959) to study the validity of their models for studying toxicant interactions. The results to be discussed involved tests of the model using lethality (Anderson and Weber 1977), growth (Koikemeister and Weber 1979; Muska and Weber 1977, Weber and Muska 1977) and on an organ system (Shelton and Weber 1981).

The regulatory agents at the beginning of our work essentially followed the National Technical Advisory Committee's recommendation that the sum of the ratios of the measured concentration of the permissible level of each toxicant should not be greater than one. This basically follows the concept of a "Toxic Unit". The "Toxic Unit" method arbitrarily assigns a value of one to that concentration which induces particular response, such as LC_{50}. The concentration of each toxicant in a mixture is then expressed as a fraction of its corresponding LC_{50} value. The fractions are added and if the resulting quantity is equal to the toxic unit (1) than a 50% response is predicted for the mixture. The basic assumption of the "toxic unit" is that each toxicant contributes to a common effect in proportion to its relative potency. In Bliss' model this would be "similar joint action" or in Hewlett and Plackett's it would be "simple similar".

A multitude of terms have been suggested to describe the various types of combined toxicant effects. Ariens (1972) and Fedeli et al. (1972) reviewed the various terminologies that have been used. As Sprague (1970) and Warren (1971) point out, the nomenclature is confusing particularly since certain terms have been defined in more than one way by different authors. Furthermore, terminology describing the mechanisms of toxicant action is not appropriate for studies evaluating the effects of toxicant mixtures on whole organism performances without knowledge of the action of the individual toxicants. To avoid both ambiguities in terminology and assumptions implying knowledge of sites and mechanisms of toxicant action, Anderson and Weber (1977) introduced the terms concentration and response addition which are mathematically analogous to the "simple similar" and "independent action" defined by Plackett and Hewlett (1952).

Concentration addition is mathematically defined as the additive effect determined by the summation of the concentrations of the individual constituents in a mixture after adjusting for differences in their respective potencies. The primary assumption governing this type of addition is that the toxicants in a mixture act upon similar biological systems and contribute to a common response in proportion to their respective potencies. Bliss (1939) and others have assumed that if two toxicants act similarly the variations in susceptibility of individual organisms to the toxicants are completely correlated. As a consequence, the dose response curves for the components and the mixture are parallel. This has been observed for some toxicant mixtures; however, Plackett and Hewlett (1952) presented examples of chemically related insecticides which gave nonparallel lines. They and other toxicologists (Ariens and Simonis, 1961) have stated, and we believe rightfully so, that parallelism and hence complete correlation of individual susceptibilities is not a necessary prerequisite for this type of addition.

In cases where the dose response curves for the individual toxicants in a mixture are parallel, a dose response curve for the mixture can be calculated based upon the assumption of concentration addition. With the regression equations for the individual toxicants in the form of $y = a + b \log x$ (where y is the % response to each toxicant and x is its concentration), the regression equation for a binary mixture can be represented by (Finney, 1971):

$$y_m = a_1 + b \log (\pi_1 + p\pi_2) + b \log Z \qquad (1)$$

where,

y_m = % response to the mixture
a_1 = y intercept of the first toxicant
b = common slope
π_1 = proportion of the first toxicant in the mixture
π_2 = proportion of the second toxicant in the mixture
p = potency of the second toxicant relative to the first
Z = concentration of the mixture

This equation can readily be adapted to represent mixtures containing more than two toxicants. It should be noted that equation (1) for concentration addition is similar in principle to the toxic unit method used by Lloyd (1961) and Brown 91968). Whereas the toxic unit method measures the toxicity of mixtures only at particular levels of response (LD_{10}, LC_{50}, etc.), equation (1) incorporates the entire dose response curve.

Response addition is the additive effect determined by the summation of the responses of the organism to each toxicant in a mixture. This form of addition is based on the assumption that the toxic constituents of a mixture act upon different biological systems within the organism. Each organism in a population is assumed to have a tolerance for each of the toxicants in a mixture and will only show a response to a toxicant if the concentration exceeds its tolerance. Consequently the responses to a binary mixture are additive only if the concentrations of both toxicants are above their respective tolerance thresholds. However, for quantal responses the tolerances to the toxicants in a mixture may vary from one individual to another in a population; therefore, the response of the test animals depends also upon the correlation between the susceptibilities of the individual organisms to the discrete toxicants. For example, in order to predict the proportion of organisms killed by a binary mixture, it is necessary to know not only the proportion that would be killed by each toxicant alone but also to what degree the susceptibility of organisms to one toxicant is correlated with their susceptibility to the other toxicant.

Plackett and Hewlett (1948) recognized this statistical concept and developed mathematical models that accounted for the correlation of individual tolerances ranging from total negative to total positive correlation. If the correlation is completely negative ($r = -1$) so that the organisms most susceptible to one toxicant (A) are least susceptible to the other (B), then the proportion of individuals responding to the mixture (P_m) can be represented by:

$$P_m = P_A + P_B \text{ if } (P_a = p_b \le 1) \qquad (2a)$$

where P_A and P_B are the respective proportion of organisms responding to the individual toxicants A and B. With no correlation ($r = 0$) in susceptibility the relationship is expressed by:

$$P_m = P_A + P_B (1 - P_A) \qquad (2b)$$

In the limiting case of complete and positive correlation ($r = 1$), individuals very susceptible to toxicant A in comparison with the population will be correspondingly very susceptible to toxicant B. In this situation the proportion of animals responding the the mixture is equal to the response to the most toxic constituent in the mixture. Mathematically this is represented by:

$$P_M = P_A \text{ if } P_A \ge P_B$$
$$P_M = P_B \text{ if } P_B \ge P_A \qquad (2c)$$

For response addition, no significance can be placed on the slope of the dose response curves because the toxicants in a mixture are acting primarily upon different biological systems with varying degrees of susceptibility between organisms. Even if the regression equations for the constituents in a mixture are parallel for toxicants acting in this manner, the dose response curve for the mixture will not be linear (Finney, 1971). This will be illustrated later for two hypothetical toxicants whose dose response curves are parallel. Although the mathematical equations (2a, b, c) representing response addition are relatively simple, the statistical consequences of this type of addition are more complicated than those of concentration addition (Finney, 1971).

Terms such as supra- and infra-addition are used to describe toxicant interactions which are greater or less than those predicted on the basis of either concentration or response addition.

LETHALITY STUDIES: Anderson and Weber (1975)

Our first efforts were before we fully recognized all the assumptions in the two models we wished to use. We felt we could simply use fish of one species and we began our work. Our first lesson was that although we were environmentally exposing the fish to toxicants, the actual concentration to give a particular response varied greatly with changes in size and stock of fish (Anderson and Weber, 1975). In the initial dose response curve we corrected for size by an exponent function of weight. This approach was developed by Bliss (1936). Bliss used the following formula:

$$Y = a + b \log M/W^h$$

This expresses a linear function between survival time and dose of different sizes of silk worm larvae. W represents weight, Y the dependent variable (death of fish in our case) and M the mean daily toxicant concentration. The Y intercept "a" and the slope "b" of each dose response was calculated. An h factor of a best fitting regression and highest correlation coefficient of the dose response curve was determined using a computer program. The toxicants we used and their corresponding h factor is found in Table 1.

Slight changes in the normal distribution of a species also was recognized as having a significant effect on the slope of any dose-response curve. We therefore attempted to control all these factors by using an inbred species of guppy. We avoided sex difference by using only males.

Table 1. Toxicants and their corresponding h factor.

Toxicant	h Factor
Copper chloride	0.72
Nickel chloride	0.67
Zinc chloride	0.3
Dieldrin	0.81
Potassium pentachlorophenate	0.72

MIXTURES: Anderson & Weber(1977)

Our first attempts were with five mixtures: copper-nickel; dieldrin-pentachlorophenate-copper-nickel; copper-zinc; pentachlorophenate-cyanide; and dieldrin-pentachlorophenate. Statistically there was an apparent parallelism between the lethal response curves for copper and nickel. We assumed that as constituents of a binary mixture, copper and nickel would contribute to the mixture's toxicity in proportion to their lethal potency. We tested organisms to a series of binary mixtures of copper and nickel. The linear function computed for the observed results for the mixture was compared to the predicted linear regression by an X^2 test for goodness of fit (Figure 1). The test for goodness of fit between the observed and predicted was significant at $P=0.05$. Our model appeared to have predicted the strictly additive action of Cu and Ni.

The slopes of the response curves for dieldrin (HEOD) and potassium pentachlorophenate (KPCR) were found not to be parallel. Binary mixtures of HEOD and KPCP were tested according to the model of response addition. There was a good fit ($P<.05$) to this model. (See Table 2)

An interesting temporal relationship between the lethal effects of pentacholorphenate and dieldrin supported (Bliss 1939) the "independent joint action" or response addition hypothesis. All mortality of fish exposed to pentachlorophenate occurred in 36 hours. The effects of dieldrin after another 10 hours. The predicted lethality of response addition for each toxicant very closely aligned themselves to each time period, that is, death from pentachlorophenate before 36 hours and death from dieldrin after 45 hours with the total equal that predicted for response addition.

Mixtures of copper and zinc produced results that did not fit either response or concentration addition. The individual dose response curves for copper and zinc were not parallel. We initially tested the mixtures on the assumption that they would be response additive. The responses were greater than predicted. Literature knowledge of the actions of mixtures of copper and zinc suggested that they were additive, a test for concentration addition was made. The numbers dead were again greater than

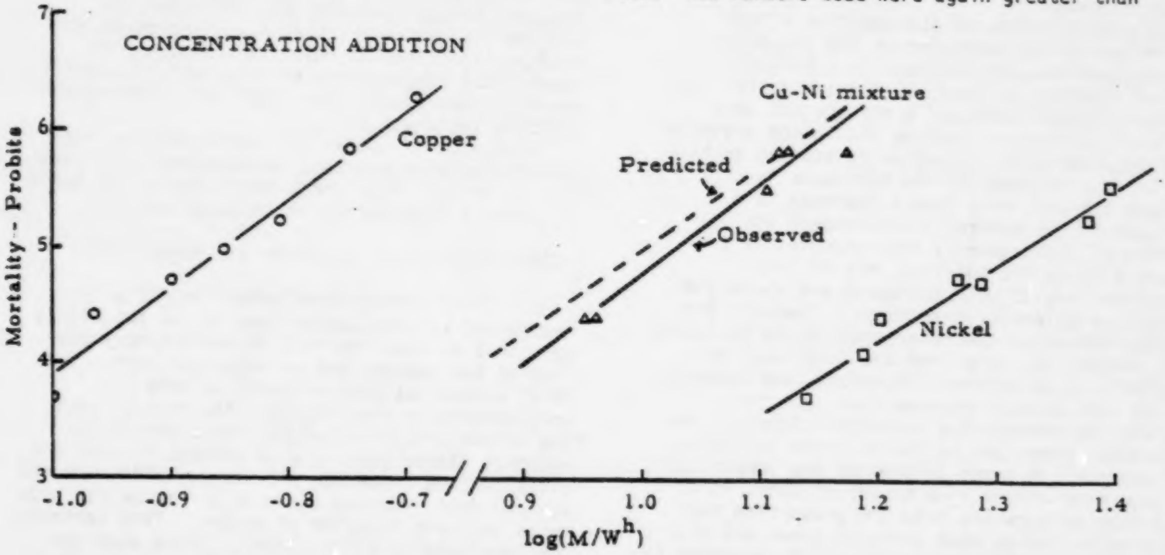

Figure 1. Lethal response curves for copper, nickel and their mixtures. The predicted regression line is based on the relative observed proportion of Cu (.006) and Ni (0.994) and a relative potency (p) of 6.58×10^{-3}.

Table 2. Toxicity study of guppies exposed to mixtures of KCP and HEOD

Assayed level of HEOD ug/l	Assayed level of PCP mg/l	Independent variable log $M/W^{.81}$ for HEOD	% Mortality predicted or HEOD	Independent variable log $M/W^{.72}$ for PCP	% Mortality predicted for PCP	% Mortality predicted for mixture $P_m=1-(1-P_1)(1-P_2)$	Observed % Mortality
5.0	0.26	-2.44	17	-0.713	11	26	10
6.45	0.40	-2.46	15	-0.639	34	44	40
6.3	0.31	-2.46	27	-0.685	17	39	50
6.4	0.40	-2.40	27	-0.58	61	72	60
4.8	0.29	-2.46	16	-0.668	24	36	70
6.9	0.41	-2.37	35	-0.594	54	70	80

From Anderson & Weber, 1977

predicted. A ratio of observed to predicted values represented a relative measure of the increased effect or what we considered to be super addition. The super addition was found to be 2.5 times above that predicted on the assumption of concentration addition.

The real challenge was to use a mixture of four chemicals, two inorganic and two organic. Nickel and copper were used as a pair that we had shown to be concentration additive. Dieldrin and pentachlorophenate were response additive. Combining the nickel and copper as a single (concentration additive) component, we treated the mixture as three response additive components (Table 3). Using the response additive approach the predicted and observed results provided a nice fit. In the case of pentachlorophenate and cyanide they were tested and found to be response additive.

We concluded that using the two forms of addition, concentration and response, we were able to describe four of the five combinations adequately using lethality as an end point. One of these mixtures contained four, two inorganic and two organic, toxicants. In the case of the one aberrant binary mixture, copper and zinc, we were able to clearly describe an interaction which is super-additive.

HYPOTHETICAL QUANTAL DOSE RESPONSE RELATIONSHIPS: Muska and Weber (1977)

Completion of these quantal studies brought us to a better understanding of the assumptions with which we were working. To illustrate graphically the relationship between concentration and response addition, hypothetical dose response curves for two toxicants (A and B) are plotted in Figure 2 expressing percent response in probits as a function of the logarithm of total concentration. In this example the dose response curves for the discrete toxicants are parallel with A being 100 times more toxic than B. We could have also chosen non-parallel curves; however, for these cases equation (1) for concentration is not appropriate. Hewlett and Plackett (1959) have developed a more generalized model (from which equation (1) can be deduced)

Table 3. Determination, using mean daily assayed concentrations, of the predicted mortality of fish exposed to mixtures of HEOD, KPCP, Cu and Ni.

Predicted Mortality Proportion $1-(1-P_{KPCP})(1-P_{HEOD})(1-P_{Cu-Ni})=P_m$	Observed Mortality Proportion	Predicted Numbers Killed	Observed Numbers Killed
$1-(1-.316)(1-.22)(1-.057)=0.50$	0.30	5	3
$1-(1-.4)(1-.36)(1-.136)=0.66$	0.60	6.6	6
$1-(1-.212)(1-.045)(1-.023)=0.27$	0.60	2.7	6
$1-(1-.268)(1-.184)(1-0.045)=0.43$	0.60	4.3	6
$1-(1-.758)(1-.198)(1-.084)=0.82$	0.80	8.2	8
$1-(1-.655)(1-.277)(1-0.081)=0.89$	0.90	8.9	9
		$X^2=5.57$	
		d.f. = 4.0	

which does not depend on the assumption of parallel dose response curves. (See Figure 2)

Dose response curves for mixtures of toxicant A and B are obtained when the total concentration is varied and the ratio of the concentrations for the individual toxicants is kept constant. Using the equations (1 and 2a, b, c) for concentration (C.A.) and response addition (R.A), dose response curves were calculated for different mixtures containing fixed proportions of toxicants A:B (1:10, 1:100, 1:1000). In Figure 2, the responses to the mixtures are shown graphically in relation to the dose response curves of toxicants A and B.

Several observations can be made from the relationships between the dose response curves in Figure 2. As should be expected, the relative toxicity of the mixture depends on the ratio of its constituents. In Figure 2, a 1:10 mixture is more toxic than the other mixtures depicted because of the greater proportion of the more toxic component - toxicant A. At certain ratios, regardless of the correlation of susceptibility (r), the relative potencies of the mixtures acting in either a concentration or a response additive manner are very similar. This is observed in Figure 2 for fixed proportions of 1:10 and 1:1000. Furthermore, for any one ratio the relative potency of the dose response curves for concentration and response addition (r = 1, 0, -1) depends on the level of response. Focusing on the dose response curves for mixtures in the ration of 1:100, it can be noted that at low levels of response (i.e., at the probit of 2 which corresponds to approximately a 0% response), the mixtures acting in a concentration additive manner are considerably more toxic than those acting by response addition regardless of the degree of correlation (r). This is due to a fundamental difference in the two types of addition. At threshold or below threshold concentrations of toxicants A and B, a mixture acting in a concentration additive manner can elicit a measurable effect because both toxicants are acting upon similar biological systems. Therefore, their concentrations can sum to produce a concentration for the mixture which is above the threshold level. However, the responses to toxicants acting upon different biological systems (response addition) are only additive if each toxicant in a binary mixture is present in concentrations above their respective threshold levels. For similar reasons, as the concentrations for the toxicants in a 1:100 mixture increase, the dose response curves for response addition (except in the special limiting case where r = 1) become progressively more toxic relative to the dose response curve for concentration addition. It is even possible that a high levels of response (in this example, for responses greater than 84% probit of 6.0) mixtures acting in a response additive manner with negative correlation of susceptibility (r = -1) can be more toxic than those acting on the basis of concentration addition.

These factors (the type of interaction, the ratio of the toxicants in a mixture, and the level of response) must also be considered along with the toxic properties of the individual toxicants in assessing the relative toxicity of a mixture. The failure to recognize these factors can potentially lead to erroneous conclusions concerning the nature of the interaction of multiple toxicants.

It is difficult to visualize the relationships between the dose response curves in Figure 2

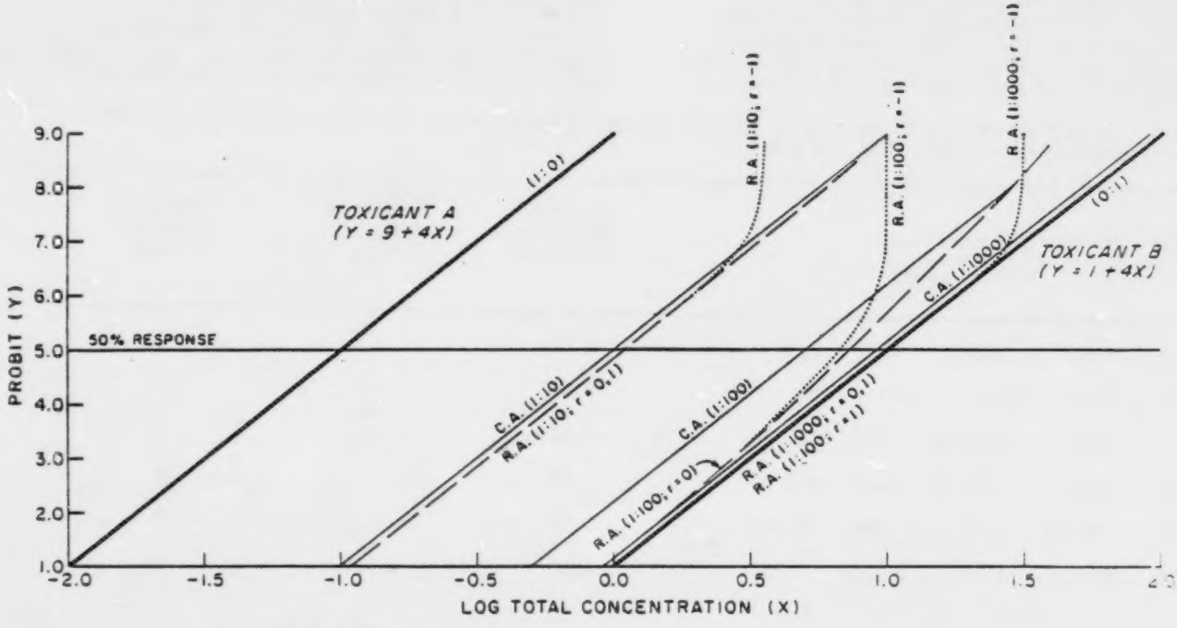

Figure 2. Hypothetical dose response curves for toxicant A (1:0), toxicant B (0:1) and their mixture containing the fixed proportions (1:10, 1:100, 1:1000). See text for explanation.

primarily due to the number of curves presented. However, the relationships between the hypothetical curve in Figure 2 can be readily conceptualized with isobole diagrams, a technique introduced by Loewe (1928, 1953). Isoboles are lines of equivalent response. They are constructed by plotting on a two-dimensional diagram the concentrations of a binary mixture of toxicants that produce a quantitatively defined response, i.e. a 10%, 50% or 90% lethal response. It should be noted that an isobole diagram can be constructed for any level or response and the relationship between the isoboles may vary depending upon the response level selected.

The isobole diagram for the 50% level of response of the hypothetical dose response curves in Figure 2 is present in Figure 3. The x and y axes in this diagram represent the concentrations of toxicant B and A respectively. The radiating dashed lines or mixing rays correspond to a series of mixtures (A:B) of fixed proportions. If the 50% response is produced by combinations of two toxicants represented by points inside the square area, the toxicants are additive. Antagonistic interactions are represented by combinations of concentrations falling outside the square.

The isoboles for concentration and response addition are determined from the concentrations of the two toxicants which correspond to the points of intersection between the 50% response line (Figure 2) and the respective hypothetical dose response curves. These concentrations are plotted in Figure 3 on the appropriate mixing ray. The lines connecting these points define the course of the isobole. Concentration addition is represented by the diagonal isobole. For quantal data, response addition is defined by the curved isoboles for complete negative (r = -1) and for no correlation (r = 0) in susceptibility. The upper and right boundaries of the square correspond to the limiting case of response addition with complete positive correlation (r = 1).

QUANTITATIVE (GRADED) RESPONSE:

A consideration of the nature of the dose response curves for quantal and graded responses shows that the effects they express are quite different. Quantal dose response curves express the incidence of an all-or-none effect (usually death) when varying concentrations are applied to a group of organisms. The curve is derived by observing the number of organisms which respond or fail to respond at various concentrations. Consequently, the slopes of these curves primarily express the individual variation of the population to a particular toxicant. Graded dose response curves characterize the relationship between the concentration of a toxicant and the magnitude of the effect under consideration. The dose response curve can be derived by measuring on a continuous scale the average response of a group of organisms at each concentration.

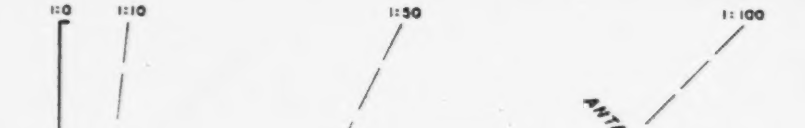

Figure 3. Isobole diagram for quantal response data. Isoboles for concentration and response addition were determined from hypothetical dose response curves in Figure 1.

As Clark (1937) and others have pointed out, it is possible to represent any graded response as a quantal response provided that the response of each individual organism can be measured. However, this procedure if adopted is at the expense of some "loss of information" (Gaddum, 1953). Quantal response data reveal only the number of organisms that respond or fail to respond at some particular concentration. On the other hand, graded response data not only tell us whether or not a group of organisms respond but also how much they respond.

The mathematical equations (2a,b,c) for the response addition are not appropriate for graded effects for two reasons. First, there is a difference in the way the two types of data are measured. For quantal responses, the proportion of organisms responding to any concentration is determined by the ratio of number of organisms showing the response to the total number subjected to the concentration. For graded responses, the mean response to each dose is measured but in general the maximum possible effect is not known, no proportional response can be calculated. This is particularly true for growth experiments where an organism's response can potentially range from growth enhancement to negative growth depending on the concentration of a particular toxicant. Secondly, the statistical concept of correlation between the susceptibilities of the organisms to the discrete toxicants in a mixture is not appropriate for graded responses measured in the manner described earlier. Graded response data represent the average response of a group of organisms. Therefore, the response of each individual organisms to the toxicants is not known. To be sure, the tolerances of the individuals in the group will vary for the different toxicants in a mixture; however, this factor will not alter the relative toxicity of the mixture because the range of tolerances of the population is theoretically represented in the sample of organisms from this population.

For graded response data, we have represented the combined response to a mixture of toxicants acting in a response additive manner as simply the sum of the intensities of response which each component toxicant produces when administered alone. A similar relationship was defined by Loewe (1953). Concentration addition can be predicted for a toxicant mixture using equation (1) if the component toxicants exhibit parallel dose response curves. Figure 4 represents an isobole diagram for a graded response. The isoboles for concentration and response addition were determined with the appropriate mathematical equations discussed.

The relatively simple types of isoboles represented in Figures 3 and 4 should only be expected for relatively simple in vitro systems or in situations where there is a clear-cut relationship between dose and effect. Given the complexity and interdependency of physiological systems, it is reasonable to suppose a priori that the special types of additivity as represented by strict concentration and response addition will be approximated only occasionally

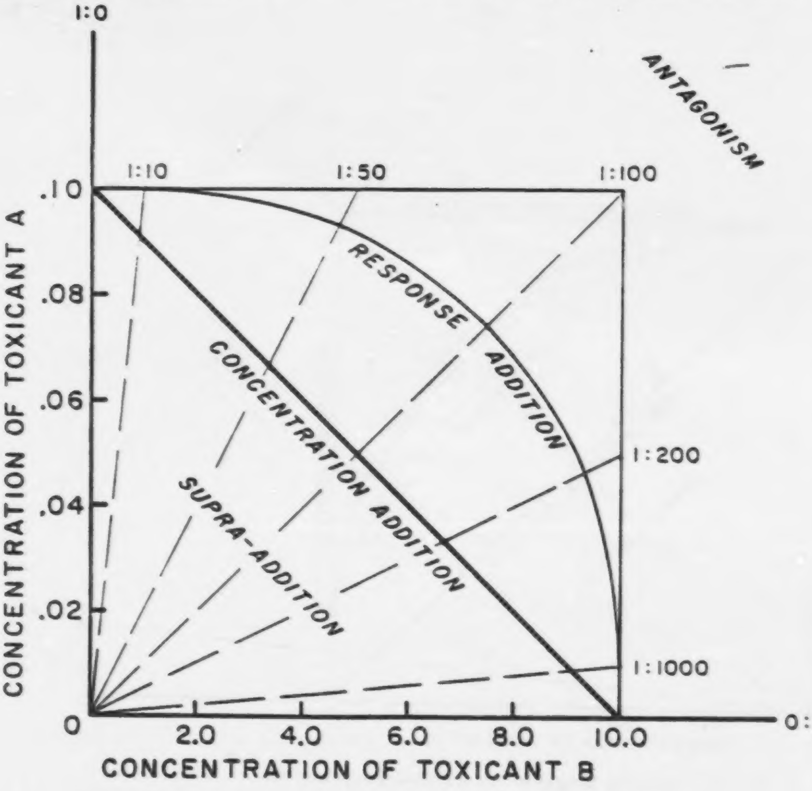

Figure 4. Isobole diagram for graded response data.

in the responses of whole organisms to mixtures of environmental toxicants. Furthermore, as mentioned earlier, the relative toxicity of a mixture depends on several factors which include the level of response (i.e., 10%, 50%, 90% response), the ratio of toxicants in a mixture (i.e. 1:10, 1:100, 1:1000), and the nature of the response itself. It should be noted that the type of addition can only be described in relation to the response under consideration. With the same mixture of toxicants, different types of toxicant interaction might be expected for different responses (i.e. survival, growth, reproduction). However, these special types of toxicant interaction do provide a frame of reference for evaluating the effects of toxicant mixtures on whole organisms performances.

Isobole diagrams are useful for visualizing the relationship between different types of toxicant interactions and for delineating the various factors which can influence the relative toxicity of multiple toxicants. However, in practice, isoboles are difficult to derive requiring a series of dose response curves for the mixture at different ratios of the component toxicants. Furthermore, there are no statistical criteria which might be used to distinguish between one form of interaction and another (Plackett and Hewlett, 1952).

GROWTH STUDIES: Muska and Weber (1977); Koikemeister and Weber (1978)

Growth was selected as the graded response for this study because it represents a performance of the integrated activities of the whole organism and as such is often a sensitive indicator of the suitability of the environment (Warren, 1971). Two of the ways environmental toxicants can affect the growth of an organisms are: (1) alter its ability to assimilate and convert food material into body tissue, and/or (2) change its rate of food consumption. To determine the manner in which toxicants affect the growth of an organism, both processes were investigated separately.

Juvenile guppies were fed daily a restricted ration of tubificid worms to determine the effect of the toxicants on the gross growth efficiency and relative growth rate (as defined by Warren, 1971) of the fish. The effect of the individual toxicants and their mixture on food consumption was investigated by feeding groups of fish an unrestricted ration and measuring the amount of worms consumed.

Parallel dose response (growth) curves were found for copper and nickel. Concentration addition was predicted as in the lethality studies (Anderson and Weber, 1977). On the basis of the mathematical model for concentration addition, the predicted dose response curves were calculated and statistically compared to the regression equations experimentally determined for the mixture. The results indicate that the effects of the copper and nickel mixture on the gross growth efficiency of the fish subjected to both the restricted (Figure 5) and unrestricted (Figure 6) feeding regimes. However, the dose response curves for the mixture representing the effects of the toxicants on the food consumption of the fish was supra-additive relative to the dose response curve predicted on the basis of concentration, Figure 7.

Dose response curves for dieldrin and nickel were accessed. Theslopes of the dose response curves for their individual effects on growth proved to statistically parallel. We judged that these compounds might interact in a response additive manner. Based on existing knowledge we assumed they should act toxicologically by different mechanisms of action. As we know the parallelism of curves is only a suggestion, not an absolute criterion for predicting either the occurrence of concentration or for the negating possible response addition. Regardless of the growth parameters we looked at the dieldrin and nickel studies were inconclusive. The reasons of course could be many. The simple model we proposed did not discriminate adequately to classify the interaction of these two chemicals.

Mixtures of zinc and nickel were tested (Koikemeister and Weber, 1978). Our assumption based upon available data and parallel growth dose response curves was that they would be concentration additive. Mixtures proved to be infra-concentration additive.

In summary the graded results indicate that the assumption of concentration addition adequately predicts the effects of a copper-nickel mixture on both the survival and gross growth efficiency of guppies. The dose response curves for the mixture representing the effects of the toxicants on the food consumption of the fish was supra-additive relative to the dose response curve predicted on the basis of concentration addition. An explanation for the differences in these two responses to the mixture was beyond the scope of the study. However, it is reasonable to assume that the effects of the toxicants on the metabolic processes involved in the conversion of food material into body tissue are different from their effects on the biological processes regulating the consumption of food.

In our studies we found that the mathematical model for concentration addition predicted the responses of guppies to both lethal and sublethal concentrations of a copper and nickel mixture. However, it should not be inferred from these results that the type of joint toxicity observed when organisms are subjected to high, rapidly lethal concentrations of mixtures will necessarily occur in cases where animals are subjected to low concentrations of the same toxicants. Furthermore, the nature of toxicant interaction can only be meaningfully described in relation to the particular response under consideration. For example, we found that mixtures of copper and nickel were concentration additive in experiments evaluating their effects on the gross growth efficiency of the guppies; however, in the food consumption studies, the same mixture at similar concentrations produced a more toxic response than was predicted on the assumption of concentration addition.

Although each of our mixtures were not accurately predicted, we must recognize that this is a simplistic model. The complexities of physiological systems from pharmokinetics to actual receptor interactions certainly makes the real world much more complex. The model does allow a specific reference point to evaluate and

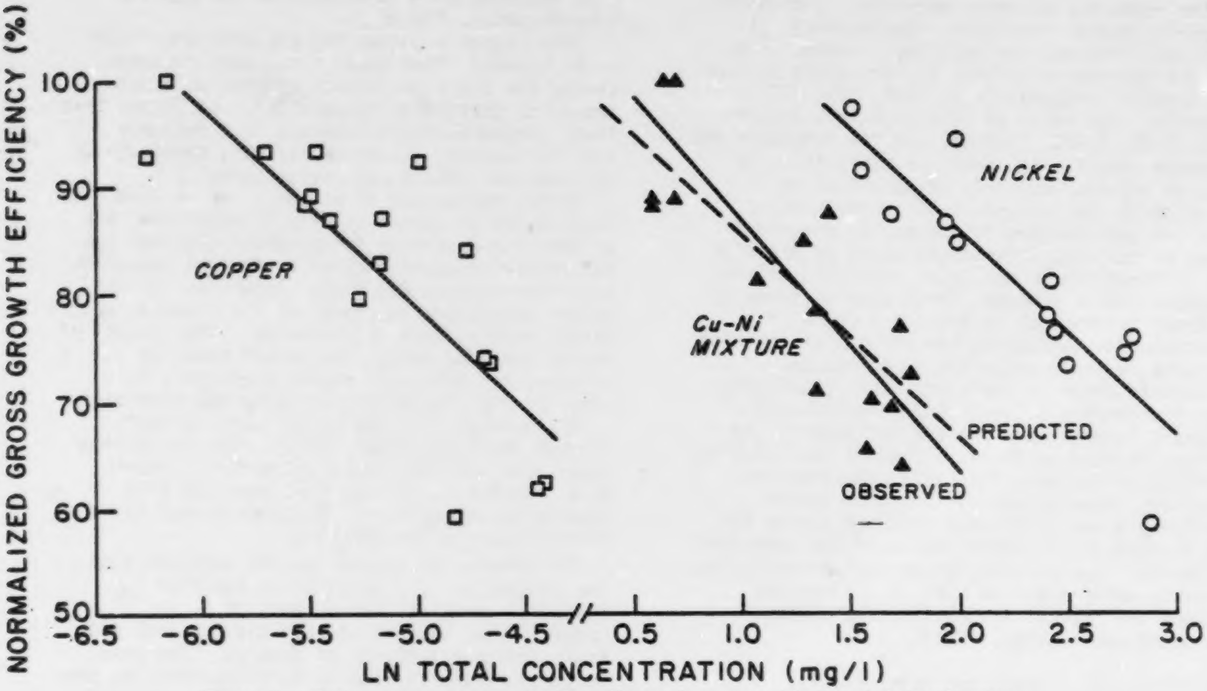

Figure 5. Dose response curves showing effects of copper, nickel, and their mixtures (observed and predicted) on gross growth efficiency normalized to responses of controls (restricted ration study).

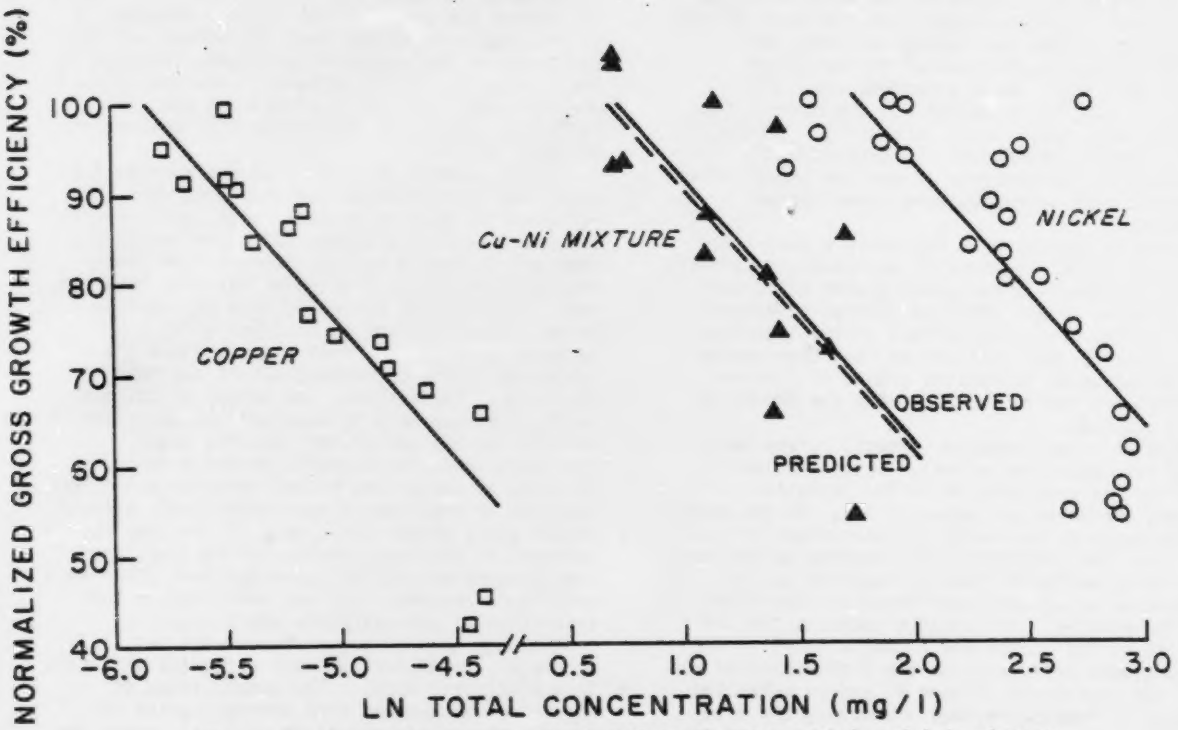

Figure 6. Dose response curves showing effects of copper, nickel, and their mixtures (observed and predicted) on gross growth efficiency normalized to responses of controls (unrestricted ration study).

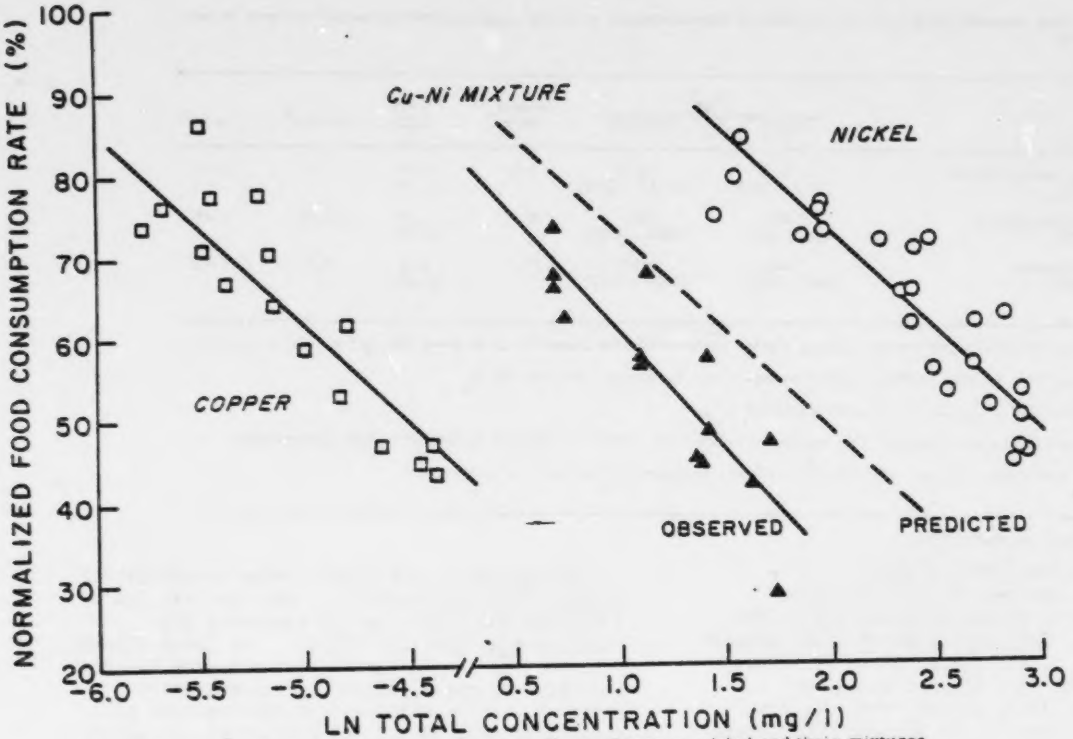

Figure 7. Dose response curves showing effects of copper, nickel and their mixtures (observed and predicted) on food consumption rate normalized to responses of controls (unrestricted ration study).

identify mixtures which deviate from the model and the direction, infra or supra-additive, from either concentration or response addition. To insure the success of a species in nature, it is necessary to evaluate the effects of potentially hazardous toxicant mixtures on the performances of whole organisms. This approach provides a methodology for assessing the toxicity of mixtures of environmental toxicants at this level of biological organization.

ORGAN SYSTEMS: Shelton and Weber (1981)

Mortality (quantal response) and growth (quantitative) response were to this point used to evaluate the concentration and response additive models for mixtures. A third test of the usefulness of this model was done using an organ system level of toxicity. The decision was made to test the model in a mammal (mice) rather than a teleost. Liver damage was the specific organ system response chosen. Plasma alanine aminotransferase (ALT, formerly GPT) activity has been shown to be a sensitive indicator of liver damage in mice (Klaasan and Place, 1966) and plasma elevation of ALT correlate well with the severity of damage (Balazs et al., 1961).

The type of joint action observed for a binary mixture can be influenced by the degree of separation in the duration of onset of toxic action for the respective toxicants in that mixture, Turner and Bliss (1953). For that reason, the temporal effects of the selected hepatotoxicants used in the study on plasma ALT were examined. All experiments were performed using male albino mice of the CF-1 strain reared in our own breeding colony and housed at five per cage. The animals weighed 25-35 g and were maintained on laboratory pellet diet and water ad libitum. The animal room was maintained at a 12-hour light/dark cycle with an ambient temperature of 70-72°F.

The toxicants were carbon tetrachloride (CCl_4), monochlorobenzene (MCB) and acetaminophen (ACET). The CCl_4 and MCB were dissolved in corn oil and ACET was dissolved in 0.9% NaCl at 40°C. The toxicants were diluted to deliver the proper dosage in a final volume of 0.01 ml per gram of animal weight. These compounds were administered intraperitoneally between 11:00 a.m. and 1:00 p.m. each day.

Liver damage was assessed by measuring plasma ALT activity. Relative plasma ALT elevations were determined at 2, 4 8, 16, 24, 48 and 72 hours following the administration of each toxicant. An optimum time interval was determined and used in the toxicant mixture study. The plasma ALT determination of Reitman and Frankel (1957) was used and the results are reported as international units (IU) per liter.

Single-component dose response curves were initially developed for each hepatotoxicant. Characteristics derived from these curves (i.e., slope, potency ration, TD_{50}) are shown in Table 4. The TD_{50}'s were used to calculate the potency ratios for the toxicants. MCB and ACET were found to be approximately equipotent in producing liver damage whereas CCl_4 appeared about 35 times

Table 4. Dose response characteristics of selected hepatotoxicants on plasma alanine aminotransferase[a] activity in male albino mice.

Toxicant	TD_{50}[b]		Potency ratio[c]	Slope (±SD)	t value[d]	P value[e]
	mg/kg	umole/kg				
1. Carbon tetrachloride (CCl_4)	16.9 (14.2 - 19.9)	109.5 (92.5 - 129.6)	1.0	6.57 (±1.19)	--	--
2. Monochlorobenzene (MCB)	428 (395 - 466)	3807 (3505 - 4136)	34.8	8.40 (±1.24)	-0.45	0.667
3. Acetaminophen (ACET)	558 (485 - 643)	3694 (3209 - 4252)	33.7	8.31 (±1.15)	-0.77	0.471

[a] A positive response is defined as a plasma alanine aminotransferase elevation ≥3 SD above the control meal (10 ± 2 IU)

[b] Determined from the dose response regression equation. Parentheses indicate 95% CI.

[c] TD_{50}(MCB or ACET)/TD_{50}(CCl_4), umole/kg comparison

[d] T value determined when slope of dose response curve for MCB or ACET is compared to that of carbon tetrachloride.

[e] In each case slopes were not significantly different from parallel at the P value indicated.

From Shelton and Weber (1981)

more toxic than either MCB or ACET. Consequently, we decided to test the joint hepatotoxic effects of the mixtures CCl_4 + MCB and CCl_4 + ACET. The large potency ratio between the constituents in the tested mixtures allowed greated resolution in differentiating the possible types of joint action resulting from them. When the slopes of the MCB and ACET curves were each compared to that of CCL_4 (t test), no significant deviation from parallelism was apparent (Table 4). It was assumed from these findings that concentration addition would be the most likely effect for the mixtures CCl_4 + MCB and CCl_4 + ACET predicted in each case.

CCl_4 + MCB Mixture. A theoretical dose response curve for the binary mixture of CCl_4 and MCB at a molar dose ratio of 1:38 was predicted using Finney's (1971) equation for concentration addition. The development of this curve involved utilization of data from the single component dose response regression equations as well as a common regression coefficient determined by analysis of covariance. This curve is shown plotted in Figure 8 along with the curves for CCl_4, MCB, and the observed curve for the 1:38 mixture. The results show no difference between the two curves at $P > 0.975$.

CCl_4 + ACET Mixture. The predictive equation for the mixture of CCl_4 + ACET was developed for a molar dose ratio of 1:36.6 (CCl_4:ACET). This curve is shown plotted in Figure 9 along with the observe dose-response curve for the mixture as well as those for the singly applied CCl_4 and ACET. The test of comparison revealed that the predicted and observed curves for the CCl_4 + ACET mixture differ ($P < 0.0005$). The observed joint effect for the mixture can thus be categorized as infra-additive on the basis of concentration addition.

To determine if response additivity might more adequately describe the observed joint effect for the CCl_4 + ACET mixture, the observe points were statistically compared to those predicted on the basis of response additivity. The findings show that the observed and predicted curves again differ ($X^2_{(5)} = 40.6$; $P < 0.0005$).

The results of the organ system investigation suggest that the toxicity of the mixtures can be predicted and classified by examining the single-constituent toxicities. The joint effects observed for the CCl_4 + MCB mixture were clearly predicted by the equation for concentration addition. It is evident that the response of a given dose of a CCl_4 + MCB mixture is not merely the sum of the toxic effects of the CCl_4 and MCB given singly. Instead, the addition of the effects follows a log-linear relationship with respect to the total concentration of both CCl_4 and MCB in the mixture.

The interpretation of the joint effects of the CCl_4 + ACET mixture is more difficult. There is an apparent antagonism exhibited with a resultant infra-additivity. Since present knowledge of the toxic mechanisms for both MCB and ACET does not present any striking differences between the two, any observed differences in joint action, when combined with CCl_4, is largely unexplained. It has been inferred that acetaminophen may damage the hepatic endoplasmic reticulum (Thoreirsson et al., 1973). If this is the case, then this could affect the activation of CCl_4, with resulting infra-additivity.

CONCLUSIONS

When we began this work we hoped to use pharmacological models already developed and apply them to problems of environmental toxicology. The desire was to have a model that had applicability to environmental problems and was rich enough in its information to lead us into an understanding of the chemicals with which we had concern. The most careful analysis of the mixtures would involve a factorially designed experiment. Using multiple toxicants and among doses a factorial design would become impossible. So the desire was to use existing knowledge and utilize the model to expand our knowledge about the toxicants. It was also our wish to have the model serve truly multiple mixtures and not just binary mixtures. We did successfully use it in a mixture of four toxicants, two metals and two

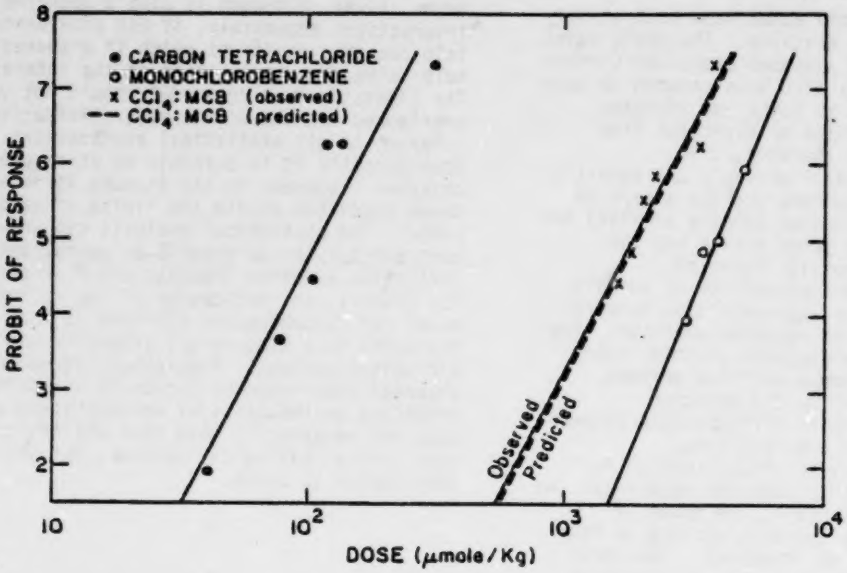

Figure 8. Dose response curves illustrating the effects of carbon tetrachloride (CCl_4), monochlorobenzene (MCB) and the 1:38 mixture (CCl_4:MCB) on the percent of animals (expressed as probit) responding with significant plasma alanine aminotransferase elevations. Both the predicted and the observed curves for the mixture are shown. Each point represents a treatment of a minimum of ten animals.

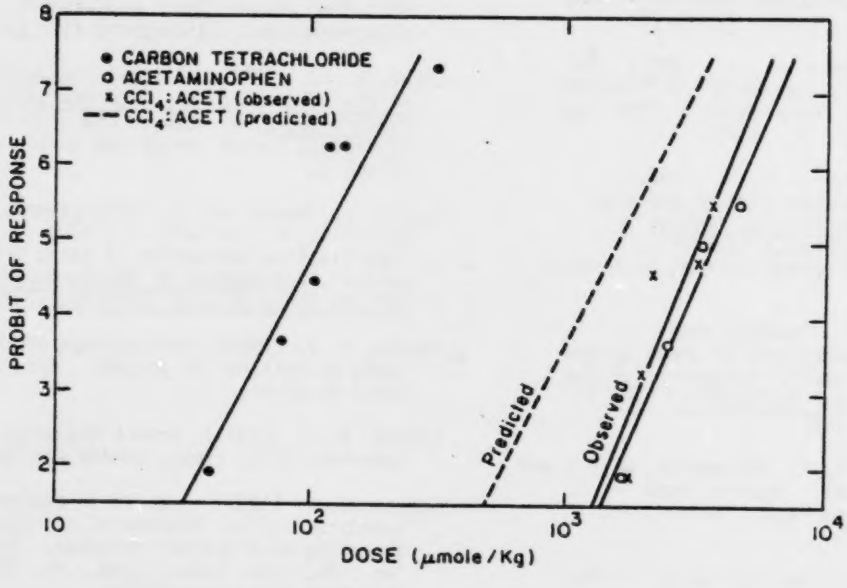

Figure 9. Dose response curves illustrating the effects of carbon tetrachloride (CCl_4), acetaminophen (ACET) and the 1:36.6 mixture (CCl_4:ACET) on the percent of animals (expressed as probit) responding with significant plasma alanine aminotransferase elevations. Both the predicted and the observed curves for the mixture are shown. Each point represents a treatment of a minimum of ten animals.

organic chemicals; on the other hand most of our tests used only binary mixtures. The basic model has been theoretically expanded upon, Christensen and Chen, 1985. It has also been reduced to such a simplistic form that it lacks any richness other than its description of direction from concentration addition, Marking, 1985.

Our own efforts using Plackett's and Hewett's (1948) noninteractive scheme was not always on the mark. The concentration (simple similar) and response (independent) joint action has the richness to describe possibilities of correlations between susceptibility of animals, interaction of infra or supra-addition compared to the concentration and response addition. The formulation of isobole diagrams plotting both concentration and response addition defined mixtures which would allow the greatest statistical opportunity to differentiate between the two noninteractive possibilities.

Our approach appears to offer a method for evaluating the effects of combined toxicants. We were successful in describing the types of interaction for binary and of a mixture of four toxicants in the case of lethality. The model also was successful in describing interactive effects of binary mixtures on growth and on an organ, liver. Although it didn't describe all interactions accurately, it did provide insight into possible questions which if answered might help solve the complexities of the interaction. The limitations of the model should not be overlooked and one of the major limitations is inherent to all statistical explanations. Statistically it is possible to state whether the observed responses to the mixture agree with those predicted within the limits of sampling error. The statistical analysis can only provide contradictory or permissive evidence, but not indicative evidence (Hewlett and Plackett, 1950). For example, an implication of the mathematical model for concentration addition is that the toxicants in a mixture act primarily upon similar biological systems. Statistical agreement of the observed dose response curves to the curves predicted on the basis of concentration addition does not necessarily mean that the toxicants act upon similar biological systems, but only that they appear to do so.

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DISCUSSION OF THE CONTRIBUTION OF L.J. WEBER AND ASSOCIATES OF THE MARK HATFIELD SCIENCE CENTER AT OREGON STATE UNIVERSITY

Bertram D. Litt, EPA/OPP/HED

The paper by Dr. Weber and his associates reviews their work of the past 12 years. They have made significant contributions to the literature on formal study of multiple simutaneous exposures of fixed mixtures of 2-4 compounds in fish and mice.

They have shown that there are limited and unpredictable circumstances in which the dose/response relationship observed fit traditional mathematical models. More important, they have demonstrated that neither structure-activity-relationships (SAR) nor parallelism of the allometric responses of individual chemicals or pairs of chemicals provide sufficient information to accurately predict the activity patterns of simultaneous exposure to three, four, or more chemicals. This is an important finding because modern man lives in an environment in which he is everywhere exposed to sophisticated combinations of chemical residues in the air he breathes, the food he eats, and frequently even the water he drinks. Weber's paper has shown that combination of toxicants can result in either superadditivity or reduction in toxicity below that predicted by simple additivity in teleosts and mammalian experimental models. This complexity precluded the use of simple strategies for dealing with complex mixtures on a routine basis. At this particular time EPA is issuing guidance recommending the use of the additivity as the fallback position for estimating cancer risk of mixtures when adequate data on the mixture is not available.

The work just summarized by Dr. Weber could be used as the first step for an ordered strategy to evaluate both fixed complex mixtures, such as pesticides where the source mixture remains constant, and varying mixtures, where concentration and constituents of pollutants vary with respect to time or distance from the source of contamination. The approach to both problems may be unified by first studying the morbidity and mortality effects of the chemical mixture at the source concentration at time t_0. A second study would repeat the initial t_0 effects as part of a series of observations to evaluate a) dimunition of effects due to temporal and/or spatial distance or b) selective deactivation of the chemical mixture.

Weber's work provides teleost and rodent models which could be used to perform rapid experiments suitable for screening chemical mixtures for the identification of components which are reinforcing and those which show antagonistic toxicity end-points. Secondly these studies should be used to identify LC_{50} levels as the initial step of evaluating the toxicity of the mixture at the source concentration and at lower concentrations of interest in rodents.

Following the screening procedure, the EPA Office of Pesticide Programs, Section F Guidelines could be followed for studying subchronic, chronic and/or teratogenic effects using the source concentration and at lower doses. The selection of lower doses should be keyed to levels indicated by physiological and environmental factors rather than the considerations listed in the guidelines for technical grade chemicals.

JOINT AQUATIC TOXICITY OF CHEMICAL MIXTURES AND STRUCTURE-TOXICITY RELATIONSHIPS

Steven J. Broderius, U.S. EPA, ERL-Duluth

INTRODUCTION

Most studies evaluating the toxicity of environmental pollutants to various aquatic organisms and systems have involved exposures to separate toxicants. Relatively few investigations have defined the adverse effects of mixtures of two or more toxicants. Effluents, leachates, and natural waters, however, frequently contain several toxic or potentially toxic substances. The zones of influence from point source pollution might also overlap. As waste treatment technology is advanced and implemented, nonpoint pollution from sources such as agriculture and atmospheric deposition will contribute to a greater degree to the overall pollutant load received by aquatic ecosystems. Therefore, in assessing the effects of toxicants on aquatic communities and to insure their success, consideration should be given to the likelihood that a wide variety of chemicals might be present simultaneously and that joint toxicity is quite likely the reason for adverse impacts of pollutants on aquatic environments.

Water quality criteria should insure that the discharge levels of separate toxic chemicals and mixtures are not deleterious to either the distribution or abundance of important aquatic populations. The setting of current water quality standards has been developed from criteria based on "no-effect levels" of single toxicants. Such a practice may be inadequate to protect aquatic organisms exposed to mixtures of chemical pollutants (Spehar and Fiandt, 1986). This practice, however, is becoming firmly established out of necessity for lack of a better approach. A tentative proposed approach to incorporate the effects of joint toxicity has been to assume strictly additive action for even diverse toxicants. Standards using this procedure, however, have generally not been set because formulating regulations on such a basis may be premature, since other forms of less toxic interaction are not uncommon.

It would be desirable to be able to predict or even estimate the probable toxic components and response for an effluent, leachate, or a water body solely from a knowledge of its important individual toxic and relatively non-toxic chemical constituents. If such a predictive approach is valid, it would be possible to determine the relative contribution of each toxicant to the overall toxicity. One could then take the appropriate action necessary to effectively reduce the toxicity of the waste water. Such an approach would better enable regulatory agencies to provide rationale for determining and predicting the effects of chemical combinations to valued aquatic organisms, and defining high hazard situations where more than one toxic substance is known to exist.

Defining the toxicity of mixtures is a major problem at both the theoretical and practical level. There has not been sufficient research to establish whether there is any widely applicable rationale and workable approach to evaluate and possibly predict the joint action of toxicants in the aquatic environment. There are a few publications (i.e., Sprague, 1970; Anderson and Weber, 1975; Marking, 1977; Muska and Weber, 1977; EIFAC, 1980; Alabaster and Lloyd, 1980; Calamari and Alabaster, 1980; Konemann, 1981b; Hermens et al., 1985; Broderius and Kahl, 1985; Spehar and Fiandt, 1986) that summarize and review much of the information on combined effects of mixtures of toxicants on aquatic organisms and approaches used to evaluate these effects. It is apparent from these articles that additional work must be conducted to characterize the joint action of multiple toxicants, especially at sublethal levels. This paper summarizes an approach to explore basic principles which govern the toxicological issues pertaining to the joint action of multiple toxicants.

TERMINOLOGY AND MODELS

Various terms and schemes for classifying and naming effects of chemicals to describe the response of test organisms to two or more toxicants, as predicted from the separate toxicity of the individual substances, have been recommended. The different forms of joint action have been graphically illustrated and discussed by Sprague (1970), Muska and Weber (1977), and Calamari and Alabaster (1980).

The development of predictive methodology to describe the joint action of multiple toxicants has been approached in two distinct ways (Marubini and Boranomi, 1970). The first approach has been to describe responses resulting from constituent interaction and to try to give them a mathematical expression based on statistical considerations. The second approach has been to postulate a physical mechanism of interaction at receptor sites, to derive theoretical response curves on the basis of assumed primary mechanisms, and to relate experimental and theoretical results. It is the general belief that the first approach is more suitable for a broad and quantitative evaluation of the joint toxicity of chemical mixtures to whole organisms. Given the complexity and interdependency of physiological systems, however, it is reasonable to suppose that a classification of the interactions between environmental toxicants into various types of responses for whole organisms will not always be possible. The real value of designating special types of toxicant interaction is that they provide a frame of reference for the systematic documentation and empirical evaluation of multiple chemical effects.

Central to an analysis of joint action are the concepts of similarity and interaction. These ideas were first proposed by Bliss (1939) for two substances and later developed by Plackett and Hewlett (1948, 1952, 1967) and Hewlett and Plackett (1959). Considering these general biological phenomena, the different types of combined effects can be identified from the relative toxicities of the individual constituents. The types of joint action are defined as similar or dissimilar depending on whether the sites of primary action to the organisms are the same or different, and as interactive or non-interactive depending on whether one toxicant does or does not influence the biological action of another.

Interactive joint toxicity is not directly predictable from the toxicity of the separate components. Models describing quantal responses to mixtures of interactive toxicants are very complex and are not described by simple formulas (Hewlett and Plackett, 1959, 1964). Certain parameters required for the models are also normally unattainable when evaluating the effect of a number of toxicants on whole organism responses. Therefore, virtually all investigators evaluating the effects of toxicant mixtures on parameters such as survival, growth, and reproduction of aquatic organisms, only consider the special cases of non-interactive joint action.

These concepts in conjunction with concentration- response curves and isobole diagrams of joint action have been used in an approach to study the lethal and sublethal toxicity of mixtures to freshwater organisms. The resulting models are named concentration and response addition (Anderson and Weber, 1975), which correspond to the previous terminology of simple similar and independent joint action (Bliss, 1939), respectively.

With concentration addition the toxicants act independently but produce similar effects so that one component can be expressed in terms of the other after adjusting for differences in their respective potencies. Even sub-threshold levels for mixtures of many toxicants can combine to produce a measurable effect. Since the toxicants act upon the same or a very similar system of receptors within an organism, the toxicants are completely correlated so that no coefficient of association need be determined. Therefore, for homogeneous populations concentration-response curves for individuals exposed to separate toxic constituents and corresponding mixtures of similar chemicals, or ones which act similarly, are expected to be parallel or similar in shape. Parallelism of concentration-response curves and complete correlation of individual susceptibilities, however, are not a requirement for this type of interaction. In cases where the concentration-response curve for the individual toxicants are parallel, Finney (1971) and Anderson and Weber (1975) have provided a procedure to predict a concentration-response curve for the mixture based upon the assumption of concentration addition. The toxic unit model (Sprague, 1970), which measures the toxicity of mixtures only at particular levels of response, can be considered a simplification of the concentration addition model. This special case of the general model assumes that a mixture should be at a particular magnitude of toxic response when the sum of the concentrations of all toxicants expressed as fractions of each toxicant's effect concentration equals unity.

A second model of joint action, response addition, is predicted when each toxic component of a mixture primarily acts upon different vital biological systems within an organism or affects differently the same systems. Each toxicant neither enhances nor interferes with one another and contributes to a common response only if its concentration reaches or exceeds a certain tolerance threshold. Therefore, multiple toxicity effects cannot be expected when each of a mixture's components is below its respective response threshold. The tolerance of individuals exposed to a mixture of toxicants acting independently may or may not be correlated. Therefore, the response curves for each toxicant of a mixture may or may not be parallel or similar in shape. If the response curves for compounds in a mixture are dissimilar or if the modes of toxic action are known to be different for toxicants which have similar response curves, then it is proposed that the degree of response to the mixture can be predicted by summing in various ways each response produced by the separate toxicants. The proportion of individuals of a group that are expected to respond or the degree of response for each individual organism exposed to specific components and combinations exerting response addition depend upon the responses to the individual compounds and the correlation between the susceptibilities of the individual organisms to each toxicant. For mixtures of two chemicals this tolerance correlation can vary from completely positive to completely negative. Three models have been proposed (Hewlett and Plackett, 1959) for correlation of individual tolerances of -1, 0, and +1. For mixtures of many chemicals the correlation coefficient (r) is expected to vary from 0 to +1 (Konemann, 1981b). Response addition is less likely to occur than other types of action because an organism is a coordinated system (Plackett and Hewlett, 1967). Nevertheless, response addition is important theoretically for it leads to a limiting mathematical model.

The application of concentration and response additive models to mixture toxicity data has not been extensive nor have the models proven to be useful in all cases. Also, when applying these classifications to mixtures of more than two chemicals, problems might arise because the joint action of the different groups can fall under different models as additional joint actions are possible between the groups. Therefore, a mathematical description of the joint toxicity of a mixture of greater than two compounds is probably only possible for special situations where non-interactive joint action seems to be a prerequisite.

The more-than-strictly additive (synergistic) and less-than-no addition (antagonistic) joint actions are characterized by a toxicity that is either greater or less than predicted from studies on the separate toxicants. With these situations the effectiveness of a mixture cannot be assessed from that of the individual toxicants. The response depends upon a knowledge of the combined effect which is usually only experimentally determined.

The different forms of joint action can be graphically illustrated by an isobole diagram as presented in Figure 1. Isoboles are lines of equal response and can be determined for different mixtures of two toxicants where the concentration of one toxicant for a quantitatively defined response (i.e., 96-h LC50) is plotted against the corresponding concentration of the other toxicant. Mixtures of toxicants A and B in different ratios are identified by lines (mixing rays) radiating from the origin of the isobole diagram. The relationship between the isoboles may vary depending upon the response level selected. Combinations of the two toxicants represented by points within the square area correspond to responses that display joint addition. An enhanced effect to that which is strictly additive, as represented by the diagonal isobole, is more-than-strictly additive. A lessened effect to that predicted for summation is less-than-strictly additive, or shows no addition. Response addition for two toxicants with parallel concentration-response curves is defined by the curved isoboles for complete negative (r=-1) and for no correlation (r=0) in susceptibility.

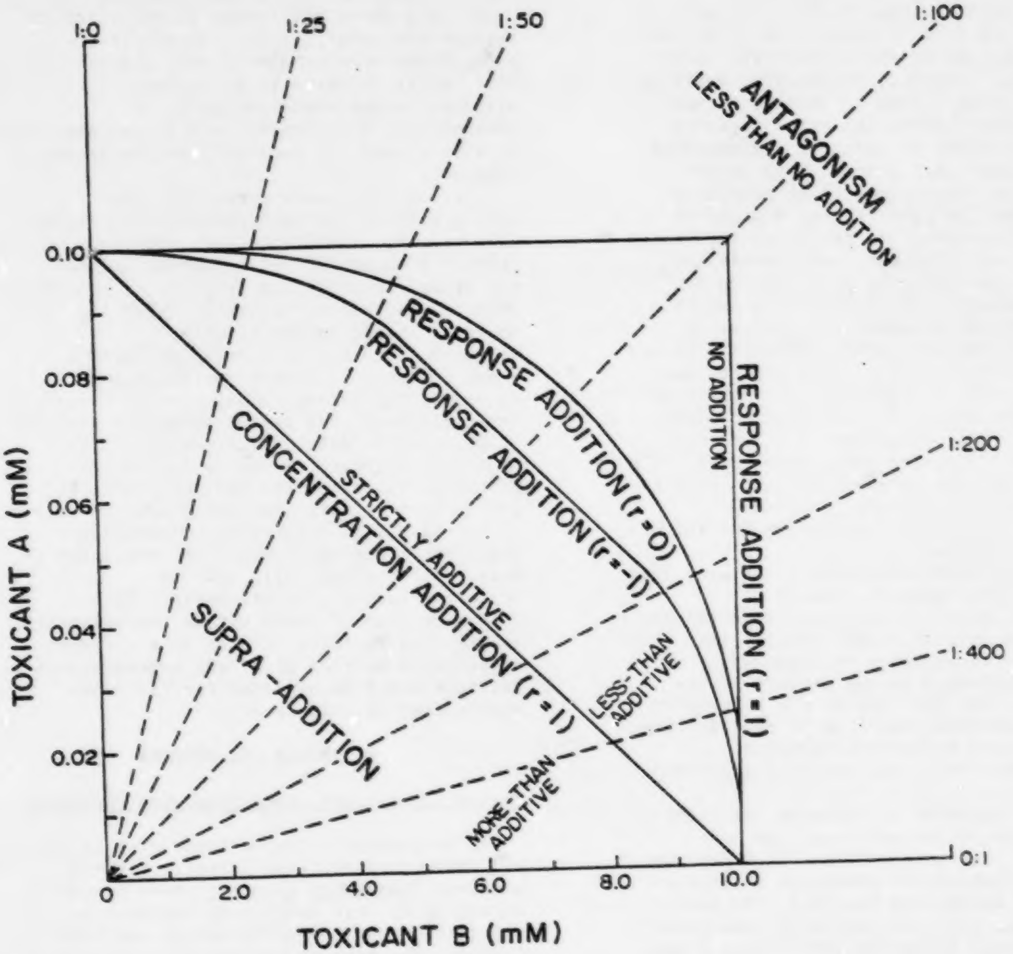

Figure 1. Isobole diagram depicting various types of lethal responses for the joint action of two toxicants displaying parallel concentration-response curves. (From Muska and Weber, 1977).

Combinations falling exactly on the upper and right boundary of the square correspond to areas of no addition or the limiting case of response addition with complete positive correlation (r=1). Areas outside of the square represent antagonistic responses (less-than-no-addition) where one toxicant counteracts or opposes the action of another beyond that expected for the individual toxicants. In a similar manner to that presented above, isobole surfaces can be defined for three toxicants. This terminology and classification scheme for the toxicity of two chemicals can, with certain modifications, be extended to chemical mixtures containing several toxicants.

EXPERIMENTAL APPROACH

A mechanistic approach incorporating toxicant-receptor theory to assess joint action has not been pursued during our research project because of the difficulty in determining and lack of understanding of the primary mechanisms by which toxicants exert their effects. Instead, the general approach has been to study the relationships between toxicant concentrations and whole organism responses which can be observed and measured.

It is proposed that general elementary principles and models describing responses resulting from toxicants having similar or different modes-of-action can guide the design of realistic and practical experiments that will provide insight into joint action of multiple toxicants. By designating special types of toxicant interaction, a frame of reference for the systematic documentation and quantitative evaluation of such effects for chemical mixtures is provided. It should be noted that the nature of each type of joint action can only be described in relation to the particular response being considered. The special case of non-interactive joint action has been investigated as a first and predominant approach to evaluating the effects of toxicant mixtures.

The specific forms of multiple toxicity that are of particular concern from an environmental point of view are characterized by those with effects either greater than or equal to that which would be expected if each toxicant contributes to the overall effect according to some function of its respective potency. Therefore, experiments were designed to differentiate between no addition, less-than, more-than, and strictly additive joint action.

The actual approach to studying the joint action of chemical mixtures must be more quantitative than qualitative. This may pose complicated statistical questions related to experimental design and analysis. To date this issue has not been adequately addressed. The experimental design should involve a wide range of concentrations (sub-threshold to effect levels for lethal and sublethal endpoints) of the toxicants alone and if possible at various proportions of chemicals in mixtures. To accommodate this need, our primary approach has been to conduct experiments to define the joint acute toxicity of binary mixtures as determined from isobole diagrams. Additional work is planned with sublethal endpoints.

Our initial experimentation has included the testing of mixtures which are expected to produce a concentration- addition response. This type of joint action in which the constituents act independently but similarily is predicted when the quantal or graded concentration-response curves for the separate component toxicants and all mixtures are parallel or similar in shape, or when the primary mode of toxic action for the test chemicals is expected to be similar. Response addition, the joint action in which the constituents act independently but diversely, can be predicted if the quantal or graded concentration-response curves for the separate component toxicants and all mixtures are non-parallel or are dissimilar in shape. This type of joint action is also predicted if there is a known difference in toxic action between the constituents. Initially, experiments were conducted with mixtures of only two toxicants with multi-chemical mixtures tested when confidence in interpreting the simpler systems was obtained or when a need for such information became apparent.

To allow for a more comprehensive interpretation and extrapolation of limited test results, a multiple toxicant study should rely on fundamental relationships between biological activity and selectivity, and the chemical nature of toxicants. Such an approach, based on quantitative structure-activity relationships (QSAR's) where toxicity is predicted from models incorporating molecular descriptors derived from structure, has been proposed by Konemann (1981b) and Hermens and Leeuwangh (1982). With this approach it can be initially presumed that chemicals causing a specific effect by a primary and common mode-of-action (i.e., narcosis, respiratory uncoupling, acetocholinesterase inhibition, etc.) can be modeled by a single high quality structure-toxicity relationship. Each different type of toxic action (selectivity) should thus be characterized by a different empirically derived QSAR, and concentration addition would be expected for toxicants within each relationship.

MATERIALS AND METHODS

Testing Conditions, Apparatus, and Procedure

The 96-h acute toxicity tests with 30-day old laboratory cultured juvenile fathead minnows (*Pimephales promelas*) were conducted according to test conditions and with an apparatus described by Broderius and Kahl (1985). The testing procedure was according to ASTM (1980). Tests were initially conducted with individual toxicants, and subsequently expanded to test solutions containing up to 21 toxicants. Seven ratios of two test chemicals were used to define the binary isobolograms with four concentrations following an 80% dilution factor at each mixture ratio.

The values for the n-octanol/water partition coefficients (log P) were taken from Hansch and Leo (1979), Veith et al. (1979), and Veith et al. (1985) or as calculated from the ClogP version 3.2 computer program developed by the Pomona College Medicinal Chemistry project (Leo and Weininger, 1984; see Leo, 1985).

Data Analysis

Data were analyzed using several statistical procedures. Estimates of the concentration of toxicant most likely to cause 50% mortality (LC50) and their 95% confidence limits were determined from relationships fitted mathematically by the trimmed Spearman-Karber method (Hamilton et al., 1977). Concentration-response slopes were determined by a least squares linear regression program.

The manner in which the combined effects of mixtures of two or more toxicants are calculated by the quantitative toxic unit, additive index, and mixture toxicity index approach have been outlined by Sprague (1970), Marking (1977), and Konemann (1981b), respectively. The procedures used to analyze results by concentration and/or response addition models are according to those proposed by Anderson and Weber (1975). A statistical procedure to determine if binary test data are better described by a straight (strictly additive) or curved isobole has been described by Broderius and Kahl (1985).

RESULTS AND DISCUSSION

Concentration-Response Curves

Acute lethality tests were conducted with juvenile fathead minnows in order to define the toxicity of individual chemicals alone and in combination with certain other test compounds. A plot was made of percentage mortality in probit values as a function of log molar toxicant concentration (Log M) for individual treatment levels from experiments conducted with several chemicals and for each of three suspected different modes of toxic action. An example of one plot is presented in Figure 2a. The slopes of the concentration response curves for each separate mode appear to be reasonably parallel and therefore can be characterized by a single slope. Plots were made of these data for the Narcosis I, Narcosis II, and uncoupler of oxidative phosphorylation model relationships, as normalized according to the potency (Log M 96-h LC50) of that for 1-octanol, phenol, and 2,4-dinitrophenol, respectively. An example of one normalized plot is presented in Figure 2b. The slope for the normalized response for each different mode is quite similar and ranges from 12.8 to 15.1 for the Narcosis II and uncoupler chemicals, respectively (Table 1). Therefore, it is apparent that the slope of acute lethality concentration-response curves cannot necessarily be used to separate chemicals by their mode of toxic action.

Isobole Diagrams

Acute toxicity tests are also conducted in order to define isobole diagrams for binary mixtures. The test concentrations of two toxicants are combined in various fixed ratios to provide seven 96-h LC50 values that define an isobologram. Results from these tests, representing three types of responses, are presented in Figures 3 - 5. Mixtures of 1-octanol and 2-octanone display a strictly additive type of joint action over the entire mixture ratio range. This is apparent in Figure 3 from a plot of the 96-h LC50 values and 95% confidence limits for the binary mixtures at 7 test ratios. A statistical analysis of these test data that establishes a

Table 1. Percentage mortality in probit values (Y) as a function of log molar toxicant concentrations (X) for 96-h acute tests with juvenile fathead minnows

Mode of toxic action	Reference chemical	Normalized concentration-response relationship (Y=a+bX)			
		Intercept	Slope	r^2	N
Narcosis I	1-Octanol	59.1 ±3.18	13.5 ±0.792	0.724	113
Narcosis II	Phenol	50.8 ±4.26	12.8 ±1.20	0.562	60
Uncoupler oxidative phosphorylation	2,4-Dinitrophenol	67.1 ±6.28	15.1 ±1.52	0.730	36

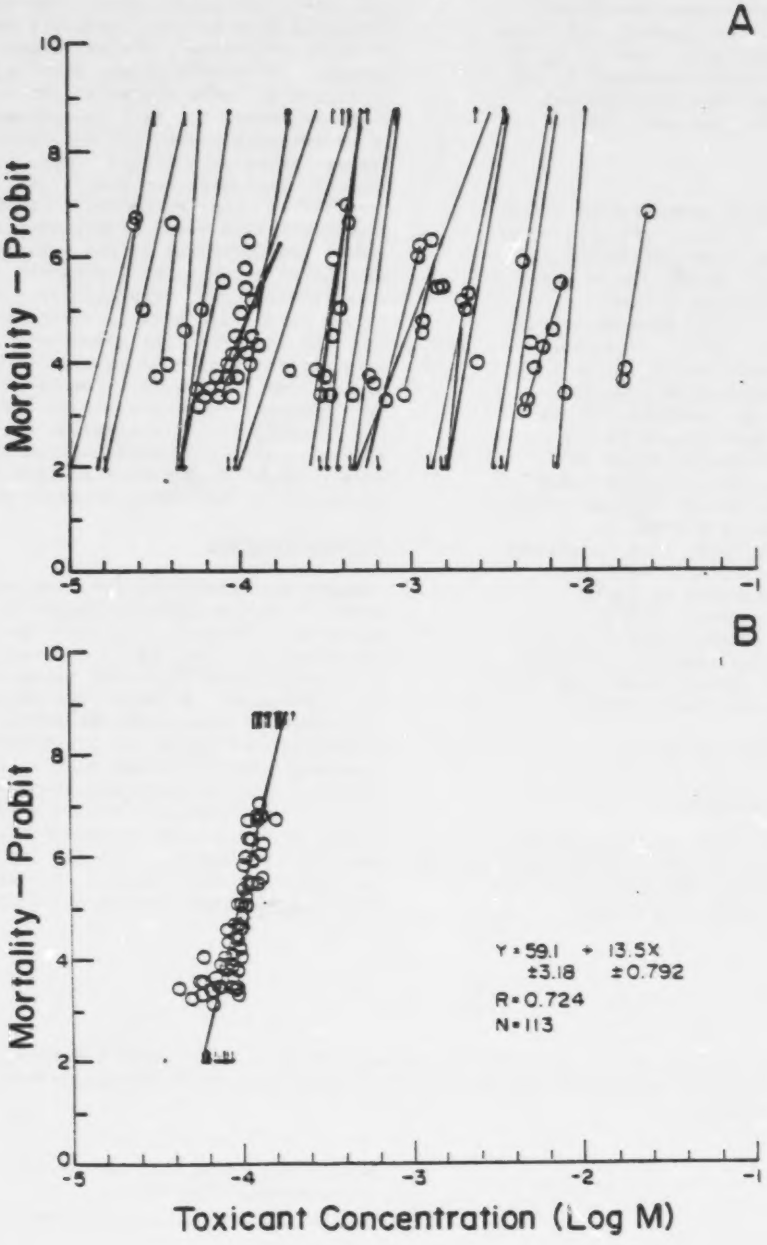

Figure 2. (A) Percentage mortality in probit values as a function of log toxicant concentration for treatment levels from Narcosis I test chemicals. Up and down arrows represent 100 and 0 % mortality, respectively. (B) Normalized plot of data in Part (A) as adjusted according to the potency of 1-octanol. (From Broderius and Kahl, 1985).

strictly additive joint toxicity was also conducted (Broderius and Kahl, 1985).

Because of the difference in symptoms associated with fish dying when exposed to 1-octanol and 2,4-pentanedione, a response additive type of joint action would be predicted for binary mixtures of these chemicals. Test results, however, were definitely not strictly additive but did show joint action that was less than strictly additive but apparently greater than response addition with r=-1 (Figure 4). Therefore, results from this binary mixture acute test did not fit either the concentration or response additive joint action models. A more hazardous joint action than response addition was observed.

The tests with binary mixtures of 1-octanol and 2-chloroethanol provided interesting but explainable results. The 96-h LC50 for 1-octanol was unchanged up to the LC50 level for 2-chloroethanol. The toxicity of 2-chloroethanol, however, was markedly reduced by the presence of octanol. It is proposed that the presence of octanol inhibits the metabolism of 2-chloroethanol to a more toxic metabolite and thus results in a complex isobole diagram. From the approximately 75 isobole diagram relationships that we have generated, the majority display a response exemplified by the first two diagrams (Figure 3 and 4). The complex type of joint action, as exemplified by 1-octanol and 2-chloroethanol, was observed in only a few of

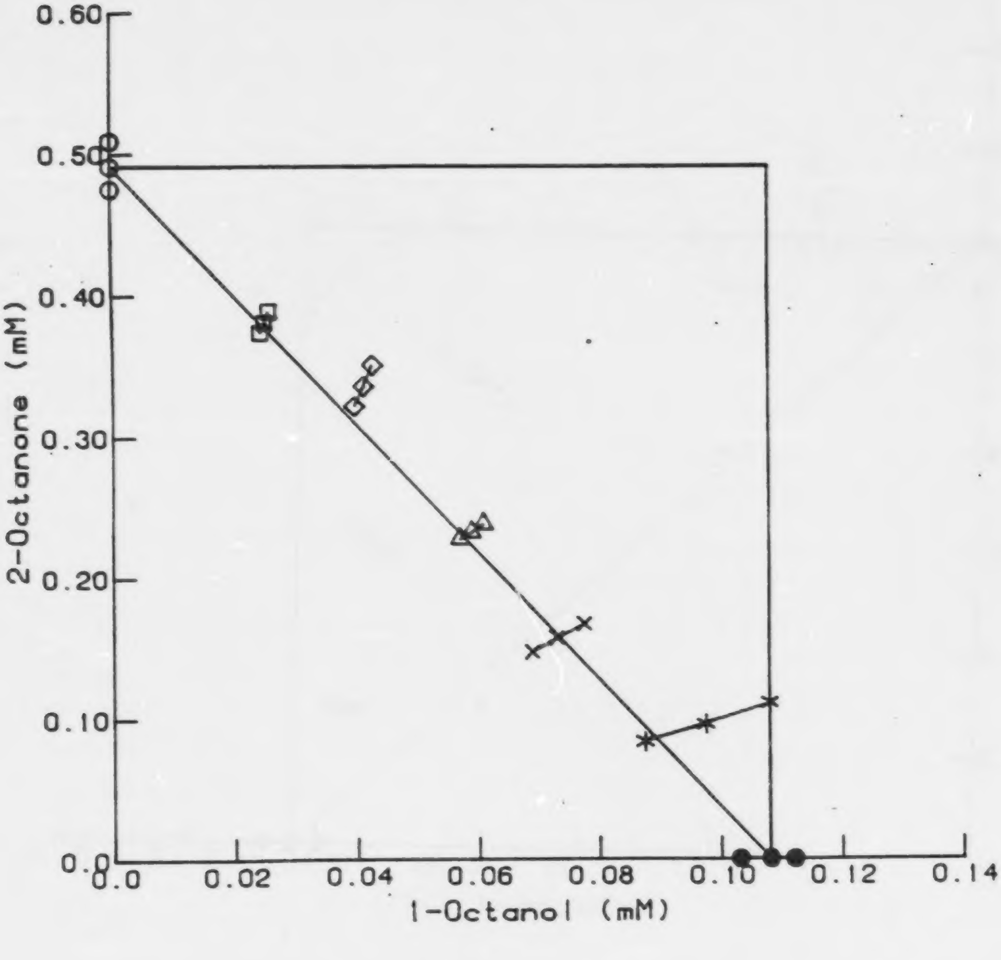

Figure 3. Isobole diagram depicting the 96-h LC50 values and confidence limits for juvenile fathead minnows exposed to different mixtures of 1-octanol and 2-octanone.

the isobole type tests. These latter tests frequently included primary aromatic amines (aniline derivatives) as one of the test chemicals. In only one instance has a markedly more than strictly additive type joint action been observed in binary mixtures of industrial organics.

QSAR and Joint Toxicity - Narcosis I Chemicals

If the results from joint toxicity tests are to make an important contribution to aquatic toxicology, a certain basic understanding as to how chemicals jointly act must be provided. Tests must also be conducted in such a manner that there is a predictive nature to our findings. To address these goals, our mixture testing effort is related to an acute toxicity data base that is being systematically generated for a program to evaluate aquatic toxicity of organic chemicals from a structure-activity approach. This data base for juvenile fathead minnows is being developed at the U.S. Environmental Protection Agency, Environmental Research Laboratory-Duluth. Some of this data has been tabulated by the Center for Lake Superior Environmental Studies (CLSES 1984, 1985). A plot of our

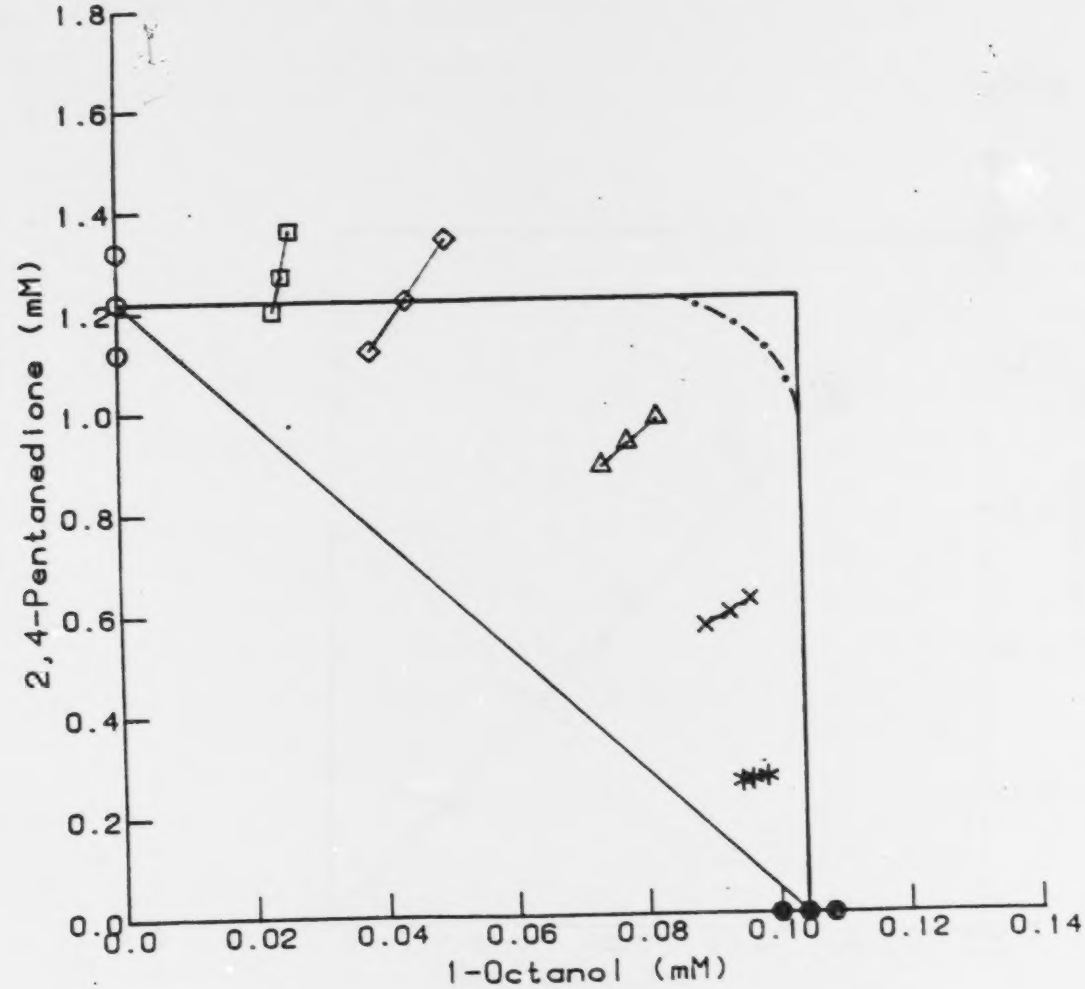

Figure 4. Isobole diagram depicting the 96-h LC50 values and confidence limits for juvenile fathead minnows exposed to different mixtures of 1-octanol and 2,4-pentanedione (-•-•- predicted relationship for response addition with r=-1).

acute toxicity data base for approximately 600 industrial organic chemicals is presented in Figure 6. The solid square data points define an approximate water solubility line above which there are very few observed data points. This line, therefore, defines a zone beyond which an acute response is not expected in a four day test. It is apparent that the data do not fall into many obvious patterns when the acute response is plotted only with log P. Virtually all of the test data fall within a log P range of about -1 to 6 and the acute toxicity is in general directly related to log P. Veith et al. (1985) observed that almost 50% of the 20,000 discrete organic industrial chemicals currently in production have log P values less than 2.0. Therefore, since our data base is representative of the TSCA chemicals, the 96-h LC50 to juvenile fathead minnows of most industrial chemicals is expected to be approximately 10^{-5}M or greater. There also appears to be a base line toxicity (Figure 6) below which a chemical can not be less toxic. This is most apparent for chemicals with a log P of less than about 4.0.

Because it is difficult to make any specific conclusions from such a plot the data were divided into smaller units and plotted according to chemical class or subgroupings. An example of one such unit was for the

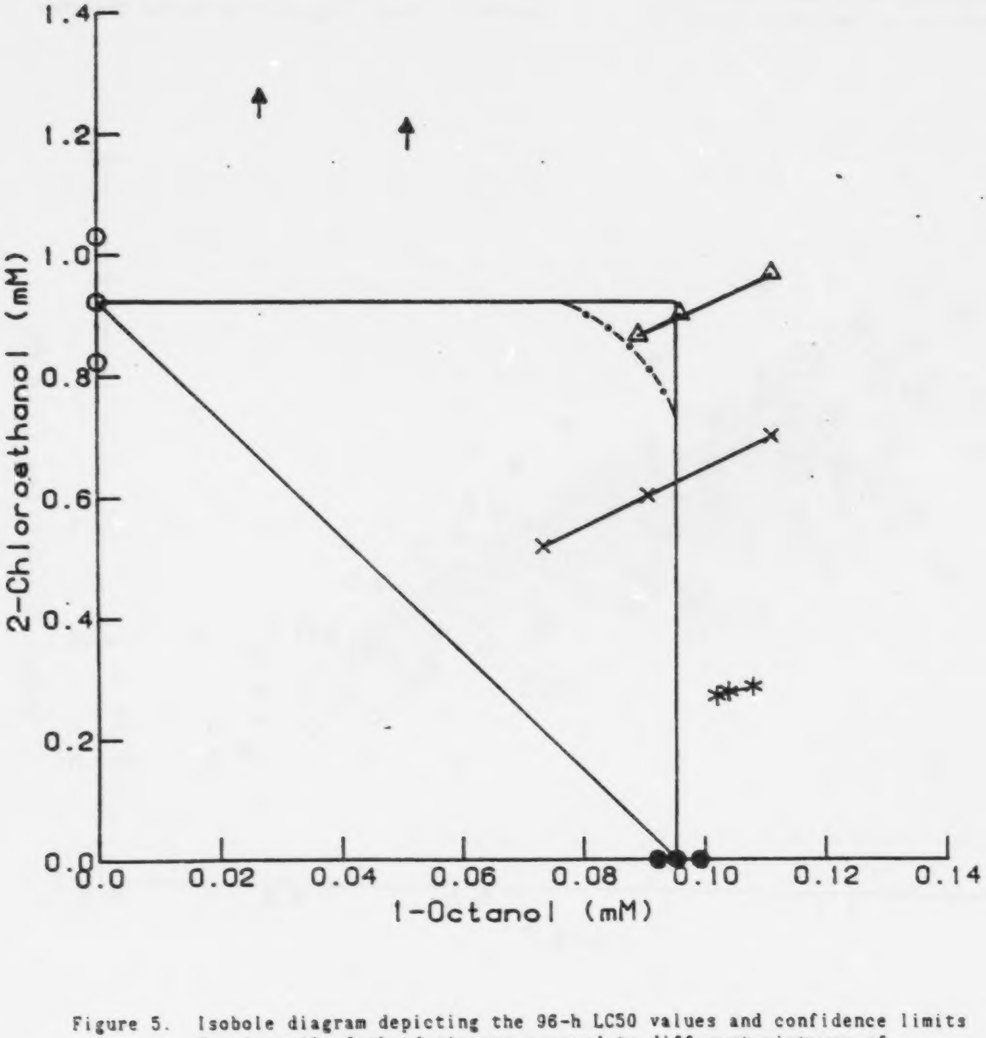

Figure 5. Isobole diagram depicting the 96-h LC50 values and confidence limits for juvenile fathead minnows exposed to different mixtures of 1-octanol and 2-chloroethanol (-·-·- predicted relationship for response addition with r=-1). Vertical arrows indicate greater than values.

ketones (Figure 7). From this plot it is apparent that the majority of the tested ketones conform to a response model line that Veith et al. (1983) have characterized by a mode of toxic action called Narcosis I. This procedure was repeated for 22 other chemical groupings and it was observed that greater than 50% of the industrial organic chemicals that we have tested conform to this non-reactive or baseline mode of acute toxic action. Therefore, the majority of organic industrial chemicals apparently do not have specific structural features which allow them to be biologically active by specific mechanisms. This nonspecific or general membrane perturbation mode of toxic action called Narcosis results from the reversible retardation of cytoplasmic activity as a result of the absorption of foreign molecules into biological membranes. The environmental concentration necessary to produce this response is independent of molecular structure and is linearly related to log P. This is only true, however, if no metabolic alterations result in more toxic metabolites and steady state equilibrium is attained.

If test chemicals are conforming to a QSAR that defines a suspected mode of toxic action, then one might expect that chemicals defining this mode will be strictly additive in their joint toxicity. To test this premise, isobole diagrams were generated for binary mixtures of 1-octanol (e.g., Narcosis I reference chemical) and a second chemical from each of seven different chemical groupings that in general conform to the Narcosis I model line. The results of these tests, as normalized to the potency of 1-octanol, are presented in Figure 8 (Broderius and Kahl, 1985). It is apparent that the isoboles are in general characterized by a diagonal line that describes a strictly additive type of joint action. This suggests that the fathead minnow perceives these chemicals as having the same

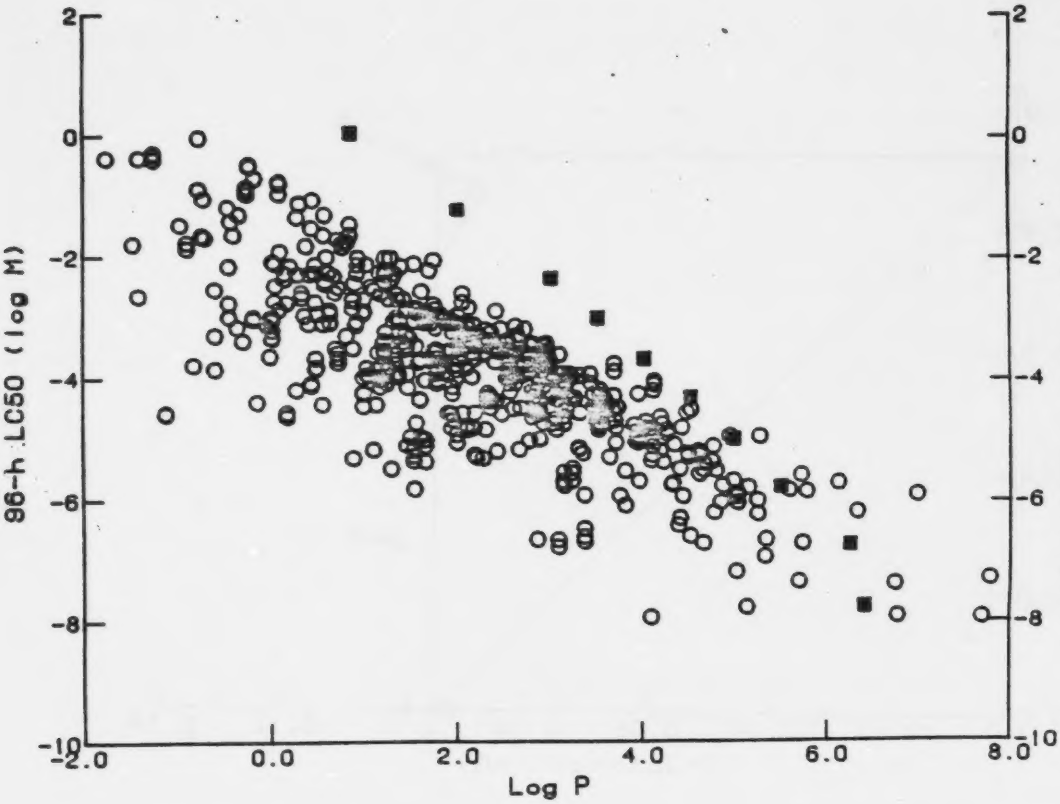

Figure 6. Acute toxicity to the fathead minnow of approximately 600 industrial organic chemicals as related to the octanol/water partition coefficient (Log P). Water solubility of alkyl alcohols indicated by square data points.

or a very similar mode of toxic action.

A second type of experiment has been conducted to document the joint toxicity of mixtures containing two or more Narcosis I toxicants. An attempt was made to prepare test concentrations of these mixtures on an equal proportion basis, based on LC50 concentrations of the individual chemicals. Using the mixture toxicity index (MTI) scale (Konemann, 1981b), it was observed that the joint action for the tested mixtures containing 2 to 21 chemicals is in general characterized by strict additivity (i.e., MTI ~ 1). Therefore, a concentration addition type of joint action has not only been demonstrated for chemicals from the same class but also for chemicals from seven different classes and in equitoxic mixtures containing up to 21 chemicals (Broderius and Kahl, 1985).

We have conducted acute toxicity tests with several alkyl alcohols, which produce a classical narcosis type of toxic action. The acute toxicity of these alcohols has been observed to increase with increasing log P and decreasing water solubility. The relationship is apparently linear for the homologs tested, with the acute response covarying with water solubility at log P values less than 4.0.

The alkyl alcohols apparently define a QSAR series when log P is used as the only independent variable. Veith et al. (1983) have proposed a bilinear QSAR model for physical narcosis that is based on a relationship derived from about 65 common industrial chemicals (e.g., alcohols, alkyl halides, ethers, ketones, benzenes). These data indicate that chemicals exerting a common narcosis mode of action, characterized by membrane expansion, may be modeled jointly, even though ethers, ketones and benzenes are in general slightly more toxic than alcohols. The joint action of test chemicals associated with the Narcosis I SAR were expected to be characterized by the concentration addition

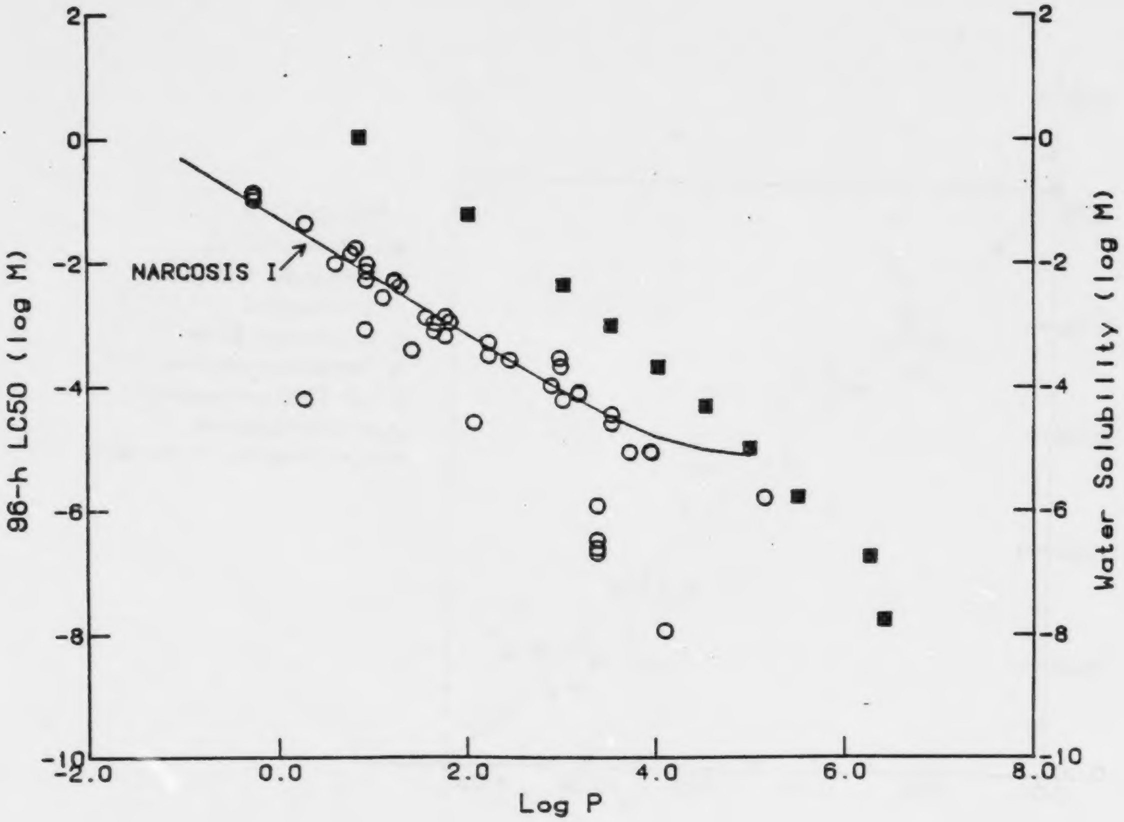

Figure 7. Acute toxicity to the fathead minnow of industrial ketones as related to the octanol/water partition coefficient (Log P). QSAR model line for physical narcosis (Veith et al., 1983).

model. Our results indicate that this was true for numerous binary and equitoxic mixtures of up to 21 chemicals.

Konemann (1981a) conducted 7 or 14 day equitoxic acute toxicity tests using guppies (*Poecilia reticulata*) and mixtures containing up to 50 industrial chemicals. Hermens et al. (1984) conducted 48-h acute toxicity tests with *Daphnia magna* and mixtures containing the same 50 chemicals as tested with guppies. When these data are plotted against the Narcosis I bilinear SAR model line of Veith et al. (1983) (Figure 9), a good log P and biological activity dependent correlation is noted among all three model lines. This suggests that the sensitivity of different fish species and daphnids to non-specific anaesthetic-like chemicals is similar since the Narcosis I model relationships in Figure 9 are all quite similar. Schultz and Moulton (1984) have recently reported a similar relationship with a different activity scale between log P and biological activity in *Tetrahymena pyriformis* for 49 aromatic industrial chemicals.

The type of joint action that Konemann (1981b) and Hermens et al. (1984) observed for mixtures containing numerous lipophilic organic compounds can generally be characterized by concentration addition. Their MTI values were reported to be 1.02 and 0.95, respectively. This was even true for an equitoxic mixture containing 50 compounds at 0.02 of their respective LC50 values. This apparent additivity for industrial chemicals characterized by a narcosis type mode of action should be of particular interest because a proportionately large number of chemicals from the TSCA inventory are likely to cause lethality through narcosis (Veith et al., 1983).

Numerous authors (Ferguson, 1939; Seeman, 1972; Konemann, 1981a; and Hermens and Leeuwangh, 1982) have suggested that physical unspecific toxicity can be minimally expected of most hydrophobic organic chemicals at some concentration. This is expected unless a chemical is metabolized or its effect is masked by overwhelming irreversible and more toxic effects from specific structural

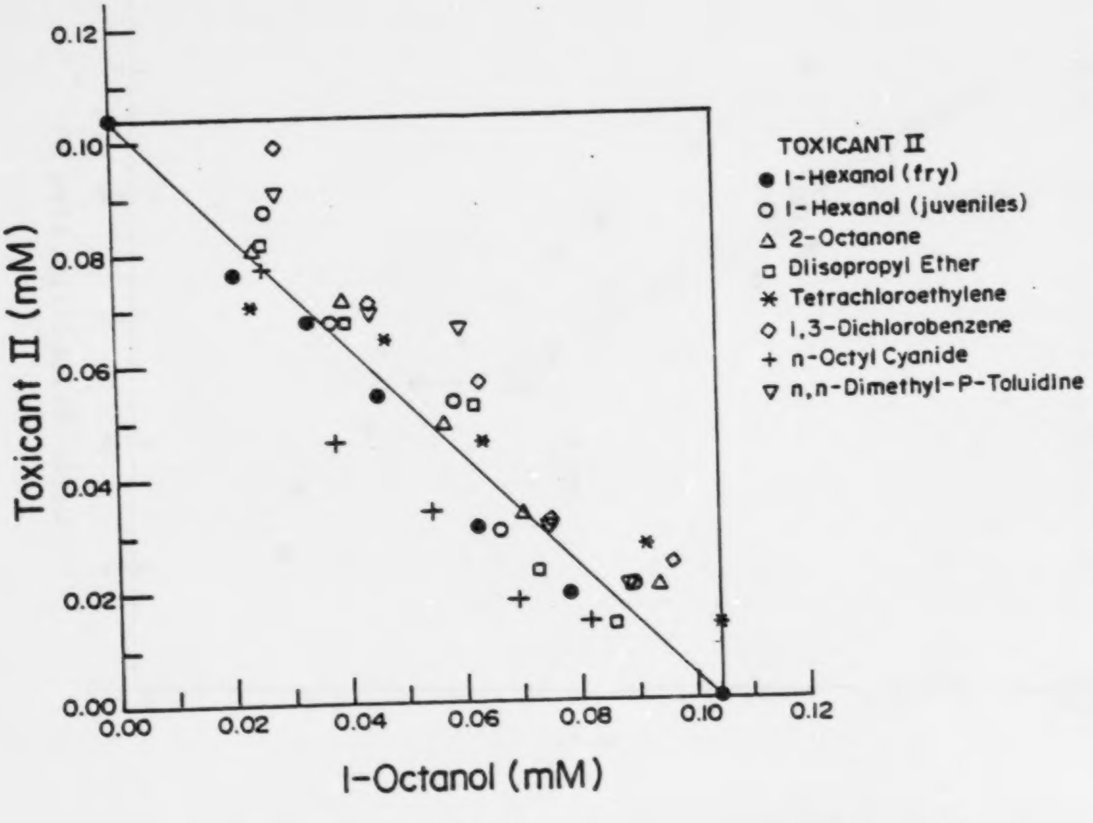

Figure 8. A composite isobole diagram of 96-h LC50 values depicting the joint toxic action for 1-octanol with seven other chemicals, each normalized to the toxicity of 1-octanol. (From Broderius and Kahl, 1985).

characteristics. In this case, a specific interaction with a receptor may be responsible for the effect. Therefore, the joint toxicity of mixtures of hydrophobic organic chemicals with various actions is minimally based on concentration addition of their minimal unspecific toxicity. This contribution of a compound in a nonionizable form can be calculated from the Narcosis I QSAR (Konemann, 1981b; Veith, 1983; and Hermens et al., 1984). In mixtures with only a few compounds with different specific and more toxic action this unspecific toxicity might not markedly contribute to the observed response. In a mixture of numerous differently acting compounds at equitoxic concentrations, the specific toxic effects might not be apparent because the concentration of the individual members will be so low. The fractional unspecific toxicity from hydrophobicity, however, will persist and this additive effect may markedly contribute to the observed response. Therefore, organic chemicals in any concentration are expected to contribute to the toxicity of a mixture with respect to the non-specific common site of action.

QSAR and Joint Toxicity - Narcosis II and Uncoupler Chemicals

There is considerable evidence that reversible narcosis might result from several mechanisms. Veith et al., 1985 have suggested that the comparatively non-specific narcosis from membrane expansion might be separated by a QSAR from narcosis by membrane depolarization. This latter more sensitive mechanism, which is observed at chemical

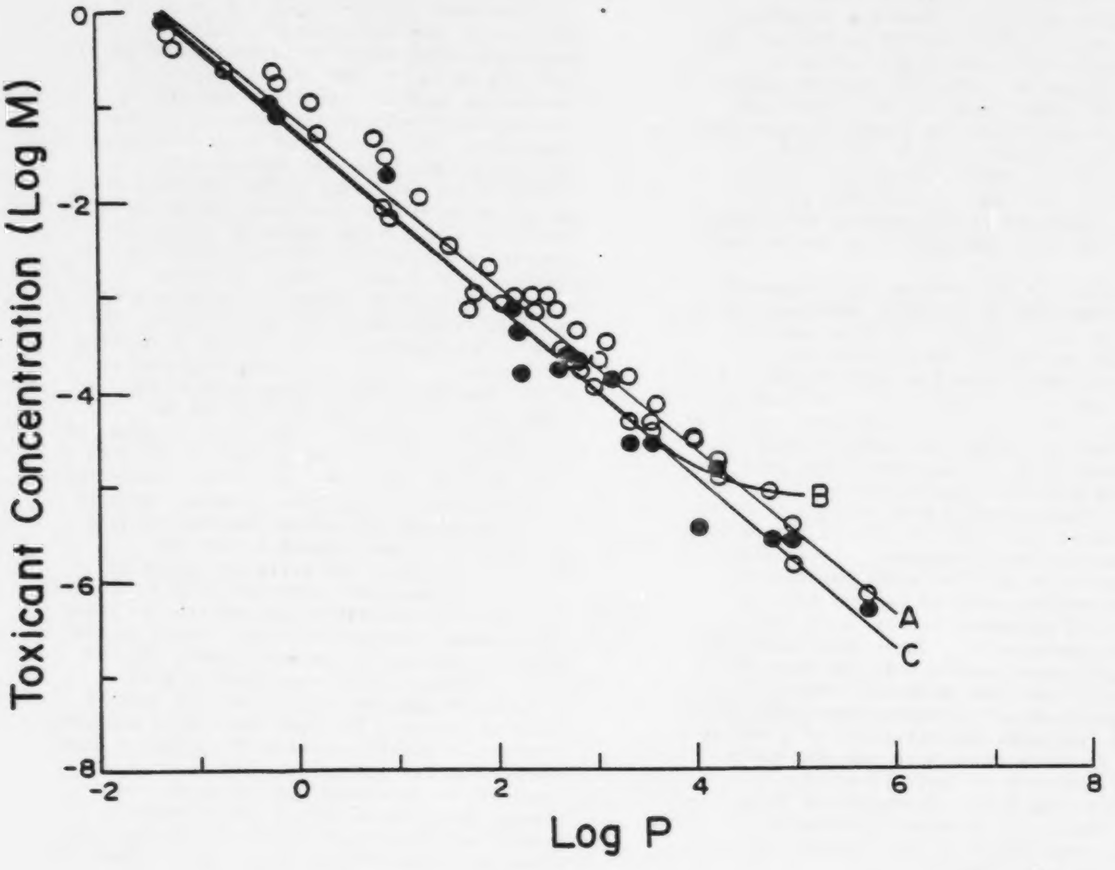

Figure 9. Acute toxicity to the guppy (0,7- or 14-day LC50) and Daphnia magna (●, 48-h LC50) of 50 and 19 industrial chemicals, respectively, as related to the octanol/water partition coefficient (Log P). QSAR model lines for physical narcosis were determined by (A) Konemann, 1981b; (B) Veith et al., 1983; and (C) Hermens et al., 1984. (From Broderius and Kahl, 1985).

activities statistically lower than the baseline narcosis (Narcosis I), is identified by Veith et al., (1985) as Narcosis II. One major class of chemicals thought to produce narcosis by depolarizing membranes at chemical activities lower than baseline narcosis is the esters (Veith et al., 1985). This group includes the benzoates, adipates, phthalates, simple salicylates, and alkyl acid esters. We cannot, however, confirm that these esters are acting by this second mode of toxic action. In fact, we have observed that many of the monoesters are approximately strictly additive with 1-octanol in their acute joint toxicity and therefore presumably act by a similar Narcosis I mode of toxic action. Several diesters were observed to be less than strictly additive with 1-octanol or phenol and thus assumed to have a different mode of action than either reference chemical.

Additional groups of chemicals that we have tested include the substituted and halogenated phenols. These compounds can generally be thought of as not chemically or biologically reactive. However, depending upon the substituents present on the molecule the hydroxyl derivative might ionize to various degrees at different test pH values. The hydroxy substituent can also conjugate with electron-withdrawing groups by resonance through the aromatic ring of the molecule (Hansch and Leo, 1979). Therefore, it was anticipated that non-log P related effects might be important in determining their toxic response and thus not modeled by the Narcosis I QSAR.

The results of our studies have suggested that the toxicity of phenolic compounds can be modeled by three QSAR's. We have observed that those non-acidic substituted and halogenated phenols with a log P of about 3 or greater are strictly additive with 1-octanol or phenol. Those phenolic compounds with high log P values are highly halogenated and/or alkyl substituted and act chemically more like hydrocarbons or halogenated hydrocarbons than phenols. Those phenols with a log P of ~3 or less, however, are only strictly additive with phenol and not with 1-octanol. Since phenol is not strictly additive with 1-octanol we feel that we have defined another mode of toxic action characterized by Veith et al. (1985) as Narcosis II. These polar chemicals are slightly more active than the baseline toxicity of non-ionic narcotic chemicals.

Multiple chemical mixtures consisting of 11 phenolic compounds characterized by a Narcosis II mode of action have been observed to be strictly additive in their joint acute toxicity to the guppy (Konemann and Musch, 1981). Their test chemicals consisted of phenolic compounds with log P values of both greater and less than 3.

A third SAR grouping has been identified and is characterized by acidic phenols. Chemicals in this group have activities lower than that of Narcosis I and II SARs and are structurally characterized as having strong electron withdrawing substituents adjacent to a hydrogen bonding group. Their mode of toxic action is thought to be that of uncoupling of oxidative phosphorylation. In our experiments we have designated 2,4-dinitrophenol (2,4-DNP) as the reference uncoupling agent for this mode of toxic action. Acute toxicity tests have been conducted with 2,4-DNP and chemicals such as HCN or rotenone which are known to inhibit electron transport in the mitochondria of cells. These latter two chemicals have activities lower than those of the oxidative phosphorylation uncouplers (Figure 10) and are therefore thought to have a different mode of toxic action. When rotenone was tested in combination with 2,4-DNP, a less than additive but more than response additive type of joint action was observed. When HCN and rotenone were tested in combination, however, a nearly strictly additive joint acute action was observed. These results are consistent with the proposal that chemicals characterized by different QSARs do indeed have different primary modes of acute toxic action and should not interact in a concentration additive manner. Those within a mode, however, should be strictly additive in their joint action.

It has been proposed that the QSARs for Narcosis II and uncoupling of oxidative phosphorylation might be improved through, in addition to log P, the use of molecular decriptors such as electronic and steric factors which reflect the polarity of the chemicals. The use of pKa as an electronic descriptor has been used extensively.

Hermens and Leeuwangh (1982) proposed that for mixtures with a relatively large number of chemicals with diverse modes of action a similar joint toxicity for the different mixtures will result. Thus, mixtures containing an equal number of chemicals will have MTI values which are approximately the same. This hypothesis was tested by Hermens and Leeuwangh (1982) with five mixtures of eight chemicals each, one mixture of 24 chemicals, and was demonstrated to be approximately correct. The joint response of the mixtures varied from partially additive to concentration additive. It is not likely that this unexpected high joint response resulted from simple similar action, because in some mixtures it is most probable that the chemicals actually had different modes of action. Hermens and Leeuwangh (1982) proposed that the most plausible explanation for their experimental results for lethal tests is that dependent action is the most likely type of joint action to occur when dealing with mixtures of numerous chemicals with diverse modes of action. The fact that these mixtures result in a nearly constant MTI value is most interesting but yet unexplained. It is important to determine how the size of a mixture group would affect these results. Hermens and Leeuwangh (1982) and others have adequately demonstrated that organic chemicals with diverse modes of action and at concentrations about 0.1 of the LC50 values and lower do contribute to the joint toxicity of mixtures. Therefore, no effect levels of separate chemicals may have little meaning for mixtures and probably should be established for groups of chemicals.

Future Research

The direction of future research in evaluating the environmental hazards posed by multiple toxicants should include not only the acute response but also important chronic endpoints such as growth and reproduction. The effects of an accumulated total body burden of toxic chemicals on reproductive success and embryo-larval fish survival and growth should be investigated. In addition to these traditional endpoints, future research might include the effects of multiple chemicals on cytotoxic responses such as teratogenic and carcinogenic effects.

Most aquatic multiple toxicant tests have been conducted with daphnids, various freshwater fishes, and a few other organisms. The incorporation of new test organisms and endpoints such as the African Clawed Frog (*Xenopus laevis*) to study teratogenic effects (Schultz and Dumont, 1984), and the rainbow trout embryo for carcinogenic effects (Black et al., 1985) might be desirable. Tests using endpoints other than those obtained from whole organism responses may also be instructive. These later tests may be of particular value when it is suspected that mixtures are displaying an interactive joint action with the metabolism of parent compounds playing a major role in defining observed responses.

The type of tests that are needed in multiple toxicant work include those that are systematically conducted with individual chemicals and various mixtures. One cannot over-emphasize the importance of a good data base on diverse chemicals. A specific test that has proven most valuable is the binary mixture test as conducted at several mixture ratios. Such data allows one to define isobole diagrams of joint action. This procedure has proven useful as a discriminating tool in identifying pairs of chemicals that have a suspected similar or different mode of toxic action. As testing has expanded into multiple chemical mixtures,

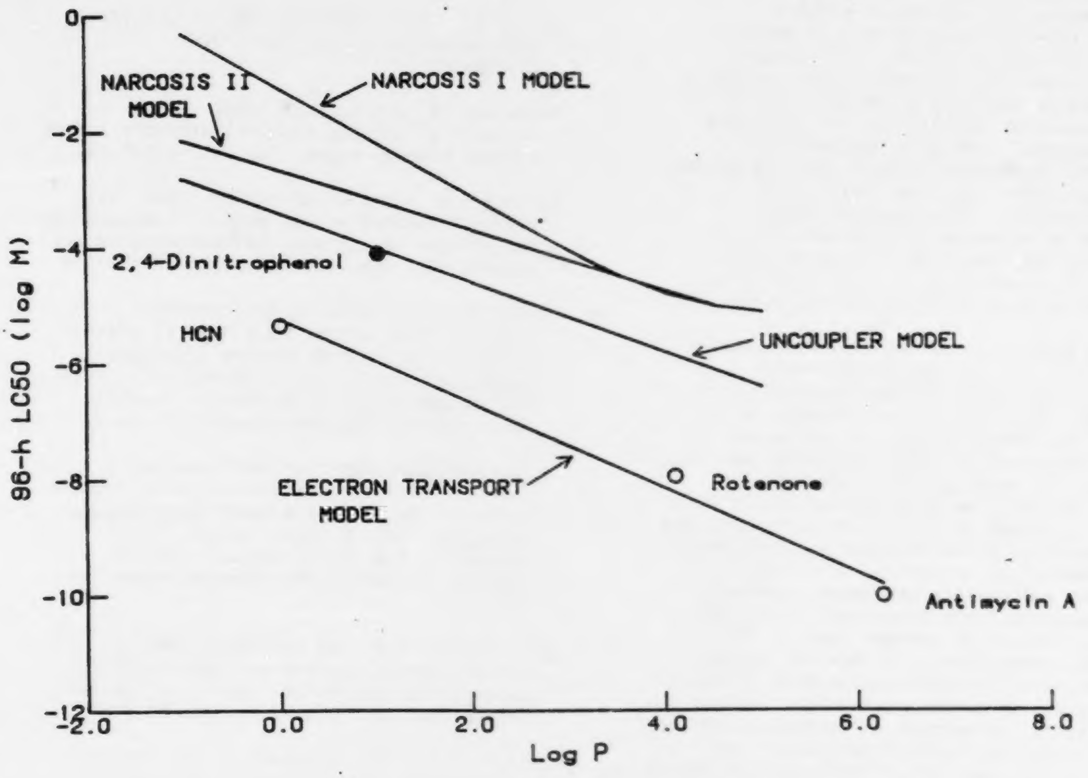

Figure 10. Acute toxicity to the fathead minnow as related to the octanol/water partition coefficient (Log P) for chemicals thought to be uncouplers of oxidative phosphorylation (●) or that inhibit electron transport and thus the metabolism of oxygen (O).

it has been the traditional approach to test equitoxic mixtures. In future testing it might be desirable to plan experiments according to a multifactorial design. With this approach all combinations of several different sets of no-effect and effect level treatments or measurements of all possible joint interactions can be tested without examining all possible combinations. The size of such studies can thus be reduced by assuming that certain interactions between the concentrations and the responses are negligible.

Our selection of test chemicals has been guided by principles established using a QSAR approach. This is done to optimize our evaluation of how chemicals jointly act and to broaden the application of test results. We have attempted to test chemicals within and between different QSAR's, assuming that we are establishing how chemicals jointly act with similar and different modes of action. Reference chemicals have been used to represent various modes of toxic action. Future experiments will include those chemicals that have a "more specific" mode of toxic action and which might display different levels of electrophilic reactivity. We have also separated our testing of organic chemicals from that of metals. It would be desirable to combine organic and inorganic chemicals into mixtures when an understanding is obtained of how each group acts separately.

The statistical analysis of our test data has been minimal. We have used standard statistical techniques as previously described by Sprague (1970), Marking (1977), and Konemann (1981b). More sophisticated techniques as reported by Durkin (1981) or Christensen and Chen (1985) might be more instructive in defining the degree of joint action and similarity among chemicals in mixtures.

Various relationships have been derived between toxicity and the octanol/water partition coefficient as the dominant parameter. This has proven adequate to describe the relationships for non-specific organic toxicants but might be inadequate for chemicals with more specific primary modes-of-action. An untested but potentially powerful approach to predicting joint toxicity of mixtures deals with N-space analysis where the "likeness" of tested and untested chemicals, and certain benchmark chemicals, can be quantitatively described. With this approach it would be assumed that if the structural properties of a chemical can be described with N factors and plotted in an N-dimensional structure space, the chemical and biological properties of a chemical should be similar to its "nearest neighbors" for which data are available. This approach might allow one to cluster compounds that show a similar mode of toxic action and thus display a concentration-addition type of joint toxic action. The type of joint action displayed by chemicals in different clusters might be characterized by a form of response addition. It is also quite probable that the type of joint action between chemicals in different clusters is too complicated to be presented by simple models and will need to be empirically defined.

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DISCUSSION

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1. INTRODUCTION AND BACKGROUND

The author deserves commendation for his paper prepared for presentation at the ASA/EPA Conference on Current Assessment of Combined Toxicant Effects to a joint audience of participants from various related disciplines. As he puts it, "defining the toxicity of mixtures is a major problem at both the theoretical and practical level ... There has not been sufficient research to establish whether there is any widely applicable rationale and approach for evaluating and possibly predicting the joint action of toxicants in the aquatic environment ... The types of tests that are needed in multiple toxicant work include those that are systematically conducted with individual chemicals and various mixtures. One cannot over-emphasize the importance of a good data base on diverse chemicals. A specific test that has proven most valuable is the binary mixture test as conducted at several mixture ratios. Such data allows one to define isobole diagrams of joint action. This procedure has proven most useful as a discriminating tool in identifying pairs of chemicals that have a suspected similar or different mode of toxic action. As testing has expanded into multiple chemical mixtures, it has been the traditional approach to test equitoxic mixtures. In future testing, it may be desirable to plan experiments according to a multifactorial design ... Our selection of test chemicals has been guided by principles established using a QSAR approach. This is done to optimize our evaluation of how chemicals jointly act and to broaden the application of test results. We have attempted to test chemicals within and between different QSAR's assuming that we are establishing how chemicals jointly act with similar and different modes of action. Reference chemicals have been used to represent various modes of toxic action has been minimal. We have utilized standard statistical techniques ... More sophisticated techniques may be more instructive in defining the degree of joint action and similarity among chemicals in mixtures ... An untested but potentially powerful approach to predicting joint toxicity of mixtures deals with N-space analysis where the 'likeness' of tested and untested chemicals, and certain benchmark chemicals, can be quantitatively described. With this approach, it would be assumed that if the structural properties of a chemical can be described with N factors and plotted in an N-dimensional structure space, the chemical and biological properties of a chemical should be similar to its 'nearest neighbors' for which data are available. This approach may allow one to cluster compounds that show a similar mode of toxic action and thus display a concentration-addition type of joint toxic action. The type of joint action displayed by chemicals in different clusters may be characterized by a form of response addition. It is also quite probable that the type of joint action between chemicals in different clusters is too complicated to be presented by simple models and will need to be empirically defined..."

The author should be complimented for his effort in developing these complex problem areas and in communicating them to the substantive scientists, statistical methodologists, and managers. The paper covers a broad spectrum of issues and approaches pertaining to aquatic ecotoxicology, risk assessment, monitoring and mangement with particular emphasis on matters relating to the perceptive isobole diagrams and the widely recognized QSAR techniques.

2. STATISTICAL CONSIDERATIONS

We initially propose to briefly discuss and formulate some of the basic statistical aspects of the approach leading to the isobole diagrams, and subsequently offer a few remarks pertaining to their role and use for field situations.

Let the toxicants be denoted by A, B, C, ... Let X_A, X_B, X_C, ... denote the tolerances of an individual to the toxicants A, B, C, ... respectively. Let E_A, E_B, E_C, ... denote the exposure/concentration levels of A, B, C, ...

2.1 Tolerance Distribution: Assume that a tolerance level can be associated with each individual organism. Thus the organism shows a response if the exposure level exceeds its tolerance. The distribution of tolerance levels across the population of individual organisms is said to be the tolerance distribution.

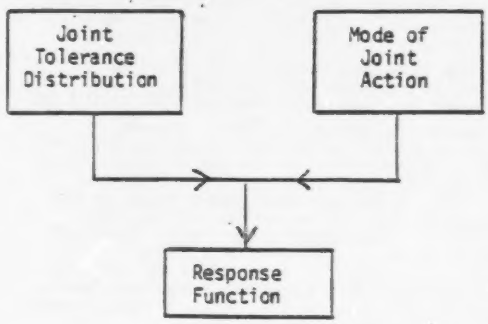
2.2 Response Function: This is the expected proportion of organisms that show a response at a given exposure level. Note that in the case of one toxicant, the response function is the same as the cumulative distribution function of the tolerance distribution. As we will shortly see, this is not true for multiple toxicants.

While the response function is directly observable, tolerances and tolerance distributions are concepts that may be useful in guiding one's thought processes. However, situations arise where the tolerance concept may be faulty. Whether a given organism exhibits a response depends upon numerous environmental factors. To the extent that these factors and their interactions are not known or are not predictable, the organism's zero-one tolerance level needs to be replaced with a "fuzzy" tolerance, i.e. there is a probability that the organism responds at a specified exposure level. The response function is then the average, taken over all exposed organisms, of these probabilities. The effect is to increase the variance or, equivalently, to decrease the slope of the probit diagram. The smaller slope is a major point of differentiation between field and laboratory investigations.

2.3 Joint Tolerance Distribution: For simplicity, we consider only pairs of chemicals and bivariate distributions. To each individual is associated a pair (X_A, X_B) of tolerances. Notice that each component tolerance, X_A or X_B, determines whether the individual responds to the chemical, A or B, when exposed to the chemical separately. There are no combined effects involved at this point. The distribution of the pairs (X_A, X_B) across all organisms in the population is the bivariate tolerance distribution. If A and B act upon similar receptor sites, then the tolerances (X_A, X_B) are expected to be positively correlated. A correlation of zero is expected if the sites are dissimilar. Negative correlation, while possible, appears to be unlikely.

2.4 Mode of Action: Unlike the univariate case, the bivariate tolerance distribution does not determine the response function. To pass from tolerance to response, an additional concept is required, one that describes the effect of the chemicals when they act in combination with one another. Let (E_A, E_B) be the joint concentration (exposure level) of the two chemicals. The mode of action of A and B should determine, in terms of the organisms' tolerances (X_A, X_B), which organisms will show a response to (E_A, E_B). Formally then, the *mode of joint action* can be defined as a rule that assigns to each joint exposure level (E_A, E_B) a region in the two-dimensional plane (X_A, X_B) of possible tolerance values. A given organism shows a response to (E_A, E_B) if and only if its tolerance pair falls within this region, which we call the *response region*.

Once the mode of action is specified, it is easily seen that the *bivariate response function*, evaluated at (E_A, E_B), is the integral of the bivariate tolerance distribution over the region associated with (E_A, E_B). *Both* the joint tolerance distribution and the mode of joint action are needed to determine the joint response function. A central issue is whether and to what extent it is possible to infer properties of the tolerance distribution and/or the mode of action from observations made upon the response function.



The mode of joint action needs to satisfy at least the following requirements (where R is the response region associated with (E_A, E_B)):

(i) The point (E_A, E_B) lies on the boundary of R. This requirement appears to rule out physical interactions between the chemicals.

(ii) If (X_A, X_B) is in R and if $X'_A \leq X_A$ and $X'_B \leq X_B$, then (X'_A, X'_B) is also in R. In words, if an organism shows a response then so will all less tolerant individuals.

(iii) If $E'_A \leq E_A$ and $E'_B \leq E_B$ then the response region associated with (E'_A, E'_B) is a subset of the response region associated with (E_A, E_B).

Figure 1 shows some hypothetical response regions that meet these requirements.

2.5 *Examples of Modes of Action*: The mode of action is called *concentration addition* when a law of simple linear substitution applies. In other words, it is possible to reduce the concentration of B and produce identical results by making a corresponding increase in the concentration of A. The response region has for its boundary a straight line with negative slope; the magnitude of the slope is the relative potency of the two toxicants. Notice that all points along this straight line determine the same response region and, therefore, the same value of the response function. It follows that, in the case of concentration addition, the isoboles (contours of the response function) are exactly the boundaries of the response regions. Concentration addition is often motivated by supposing that the toxicants act upon the same receptor sites, thereby implying a perfect correlation in the tolerance distribution. We see that a perfect correlation is not a logically necessary condition for concentration addition. In fact, there are infinitely many tolerance distributions that assign the same probabilities to the triangular response regions and thereby determine the same response function.

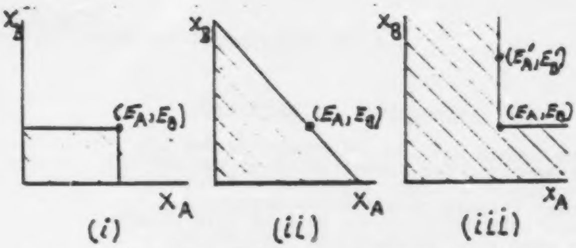


Figure 1. Response regions (shaded) for three different modes of action: (ii) concentration addition, (iii) response addition. The mode of action (i) has no standardized name but represents a situation in which A and B act upon different sites and these sites form a parallel-system in the sense of reliability theory.

From a regulatory standpoint, it is the law of substitution that is important and it is the linearity of that law that makes for a simple regulatory strategy. One can easily envision situations involving nonlinear laws of substitution (Figure 2). Let us define a mode of action to be *self-similar* if every point on the boundary of a response region R has R as its response region. Concentration addition is self-similar, as is any mode of action whose response regions have boundaries defined by single equations such as $X_A \cdot X_B$ = constant or $X_A^2 + X_B^2$ = constant.

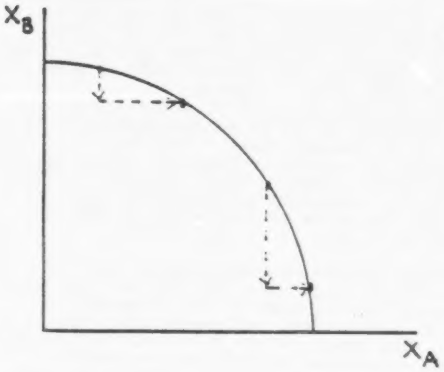


Figure 2. Example of nonlinear law of substitution. Response regions have circular arcs for their boundaries. All chemical combinations along one of these arcs produce identical responses.

In common with concentration addition, all self-similar modes of action have the two properties that (i) isoboles are the boundaries of the response regions and (ii) there are infinitely many different joint tolerance distributions that yield the same response function (in fact one can always find such a joint tolerance distribution that is perfectly correlated in the sense of concentrating its probability mass on a one-dimensional subset of the (X_A, X_B) plane).

A second mode of joint action is known as *response addition*. This occurs when an organism shows a response to (E_A, E_B) if and only if it would respond to E_A acting alone or to E_B acting alone. Response addition calls for the simple regulatory strategy of setting seperate standards for each of the two toxicants. The response region for response addition is shown in the third diagram of Figure 1. The picture reveals the aptness of the term "response addition" since the total number of responses is the sum of the responses to A and the responses to B (after adjusting for double counting).

Response addition is not a self- similar mode of action; for example, the points (E'_A, E'_B) and (E_A, E_B) in Figure 1 determine different response regions. The shapes of the isoboles depend upon the joint tolerance distribution. By contrast, for a self-similar mode of action, we need the tolerance distribution to determine the levels (LC50, LC80, etc.) but not the shapes of the isoboles. Also, in the case of response addition, the joint tolerance distribution is uniquely determined by the response function. Indeed, from Figure 1, the response function evaluated at (E_A, E_B) is $1 - F(E_A, E_B)$ where F is the survivor function of the tolerance distribution.

3. STATISTICAL ISSUES IN THE APPROACH OF BRODERIUS

This section hopes to identify a few statistical issues that seem to be implicit in the approach that Broderius has presented. This is not an exhaustive list, but only indicative and preliminary.

3.1 *Isoboles and the Nature of the Joint Action*: Isoboles are the appropriately chosen contours of the response function. They depend upon *both* the mode of joint action *and* the joint tolerance distribution. Thus, it is impossible to infer the nature of the joint action from the examination of the isoboles alone. It is necessary to know or to assume a model for the joint tolerance distribution. Broderius appears to assume a joint probit model. But different models could yield different conclusions regarding the nature of the joint action.

3.2 *Isoboles and Levels of Isoboles*: Broderius restricts attention to LC50 isoboles. Would the conclusions be qualitatively the same or different if other levels were employed? It should be helpful to investigate these problems both in theory and practice.

3.3 *Biological Homogeneity in Broderius Approach and Field Heterogeneity*: The laboratory work described by Broderius maintains a high degree of biological homogeneity. This results in the steep slopes in his probit diagrams and nearly degenerate tolerance distributions. Even within the framework of probit model, the isoboles corresponding to response addition are heavily dependent upon the slopes. It is not apparent that conclusions about modes of joint actions that are derived from laboratory studies under regimes of strict biological control could be extrapolated to field conditions, where biological as well as environmental heterogeneity prevails.

3.4 *The Issue of Synchronous and Asynchronous Exposures*: Fish are mobile, sometimes highly so, and are exposed to a variety of toxicants during their lifetimes. Would the results from Broderius study, which assume synchronous exposure, carry over to the asynchronous exposure that is common under field conditions?

4. CONCLUDING REMARKS

Steve Broderius has presented a very interesting and illuminating paper on a problem of current practical concern in aquatic ecotoxicology. It reminds us of three workshops on aquatic toxicology and risk assessment held in the recent past.

The Northeast Fisheries Center of the NOAA/NMFS organized a workshop in 1983. Issues involved definition of water management zones, grouping of chemicals and endpoints with a view to be able to consider representative chemicals and representative endpoints, and formulation of indicators and field based statistical indices leading to a crystal cube for coastal and estuarine degradation.

The EPRI workshop had emphasis on multivariate bioassay, ecological risk assessment, and relevant experimental designs.

The NOAA Chesapeake Bay Stock Assessment Committee has had its thrust on partitioning fish mortality due to pollution (multiple chemicals included), environment, habitat, and fishing that has involved multivariate multiple time series and categorical regression related tools.

Broderius' paper develops a promising approach to the contemporary issue of multiple toxicants and raises several challenging and fascinating technical problems such as: statistical graphics of combined effects, multivariate tolerance distributions, binary mixtures and multivariate results, synergism concepts for the 'whole' being 'more' than the 'sum', QSAR related chemical species grouping methods reminding one of ecological 'guilds' and functional groups, and so on.

The multiple toxicants 'ball' is not just in a statistical court. It is in every other relevant court at the same time. It will take a timely interdisciplinary effort involving simultaneous (and not sequential) collaboration of various substantive players. We wish to congratulate Steve Broderius for this interaction at this ASA/EPA Conference.

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DEVELOPMENT OF MODELS FOR COMBINED TOXICANT EFFECTS

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ABSTRACT

Adequate univariate dose-response functions are necessary in order to develop a satisfactory multiple toxicity model. We investigate here the use of univariate Weibull and probit distributions with literature data for the quantal response of fathead minnows (Pimephales promelas) to 27 different organic chemicals. We also examine fits of the Weibull, probit, and logit models to literature data for the growth rate and yield of the diatom Navicula incerta inhibited by Cd, Cu, Pb, or Zn. The Weibull model appears to provide a superior fit for both fish and algae, thus supporting a previously developed mechanistic-probabilistic basis in terms of chemical reactions between toxicant molecules and receptors of the organisms.

The application of a general multiple toxicity model is demonstrated using published experimental results regarding the action of binary combinations of Ni, Cu, potassium pentachlorophenate, dieldrin, and potassium cyanide on male guppies (Poecilia reticulata). We also analyze results of our own experiments regarding the combined effects of Ni^{2+} and Zn^{2+} on the growth rate based on cell volume of the green alga Selenastrum capricornutum. Most of the multiple toxicity data are fitted well by the model.

INTRODUCTION

Aquatic Ecotoxicology is becoming a topic of major concern (1,2). It deals with the response of aquatic organisms to toxicants such as heavy metals and organics, both in natural waters and water and wastewater treatment plants. One important goal is to protect aquatic organisms against adverse effects from pollutants.

Several factors complicate the evaluation of the toxic response of aquatic organisms to specified concentrations of pollutants. For example, the chemical form of heavy metals is important. It is well known that the ionic form of metals such as Cd, Pb, Ni, or Cu is generally more toxic than the complexed forms (3). For organics, e.g., polychlorinated biphenyls (PCB's) or polycyclic aromatic hydrocarbons (PAH's), the octanol-water partition coefficient is of interest. This is because there is often a correlation between this coefficient, the lipophility, i.e., the solubility in fat, and the toxicity (4). Other factors include volatilization to the atmosphere and partitioning to particulate matter. Considerations related to the organisms are exposure time, biomagnification, age, and species composition.

The response obtained within a given time of exposure, e.g., 96h, has been studied for many different compounds and a variety of organisms such as fish and algae (2,5). However, in most cases, only one toxicant has been considered in any given experiment. This is obviously a simplification since actual aquatic systems usually have more than one dominant toxic compound. The objective of the present work is to introduce a multiple toxicity dose-response model and apply it to fish and algae. Univariate dose-response models for these organisms will also be examined.

CLASSIFICATION OF BIOASSAYS

The response of aquatic organisms to toxicants can be evaluated from bioassays conducted in the laboratory or in the field, or in some cases, from the observation of actual ecosystems. Possible forms for laboratory bioassays are shown in Table 1 (6). For most macroorganisms, or mixed cultures of microorganisms (Groups I and II), there is a tolerance distribution for individual organisms. This means that some organisms with high tolerance will survive at high concentrations or long exposure times while others with low tolerance will not. In contrast, organisms from a pure culture of microorganisms (Groups III and IV) originate from a single clone and, therefore, have the same genetic material. Thus, there is no tolerance distribution for individuals which will respond in the same way to the toxicant.

The response can be quantal or continuous. An example of a quantal response is death for Group I organisms. A continuous response can, for example, be growth rate based on biomass (Groups II, IV). For Group I organisms, the response is the fraction of all individuals that are affected, e.g., by death. Similarly, for Group III organisms, we may consider the response to be the fraction of subsequent cell divisions that are blocked. This is the same as the reduction in relative growth rate based on cell number. This interpretation is extended to apply also to Group IV organisms.

DOSE-RESPONSE MODELS FOR ONE TOXIC SUBSTANCE

Dose-response models for a single toxicant, assuming a fixed time of exposure, e.g., 96h, are shown in Table 2. Of these, the probit model (7) is perhaps the most well known. It is based on a normal distribution of the response as a function of log(z) were z is a toxicant concentration. Other useful linear expressions are the logit transformation (8), and the Weibull transformation (9).

The probit, logit, and Weibull models must be considered mainly empirical although some

TABLE 1. Populations of Organisms Considered in Bioassays

Genetic Characterization	TYPE OF RESPONSE Quantal Response	 Continuous Response
	Group I	Group II
Tolerance Distribution for Individual Organisms	Macroorganisms Response: death of an organism Classic probit analysis Binomial statistics	Macroorganisms Mixed cultures of microorganisms Response: growth rate, C-14 uptake, respiration
	Group III	Group IV
All Organisms from a Single Clone (No tolerance distribution for individual organisms)	Pure culture of microorganisms Special case: Synchronous growth Response: growth rate based on cell number	Pure culture of microorganisms General case Response: growth rate, C-14 uptake, respiration

TABLE 2. Comparison of the Weibull Transformation with the Probit and Logit Transformations

Type	Transformation*	Probability of Response or Relative Inhibition
Weibull	$u = \ln k + \eta \ln z$	$P = 1 - \exp(-e^{u})$
Probit	$Y = \alpha + \beta \log z$	$P = \frac{1}{2}(1+\mathrm{erf}(\frac{Y-5}{\sqrt{2}}))$
Logit	$\ell = \theta + \phi \ln z$	$P = 1/(1 + e^{-\ell})$

*z is a toxicant concentration
k, η, α, β, θ, ϕ are constants; $A = \ln k$

theoretical basis has been claimed. The probit model is based on the often found log-normal distribution in biological systems. The logit model is valid for certain types of autocatalysis and enzyme kinetics (Group III and IV organisms) (10, 11). The parameter ϕ is the number of toxicant molecules per receptor. It appears that the Weibull model may have a similar interpretation so that η would be the number of toxicant molecules reacting per receptor molecule (12, 13). In addition, the Weibull model is related to the multistage model in carcinogenesis and is identical to the single-hit model for $\eta = 1$ (14).

Applications

Fish. To illustrate differences between the probit and Weibull models, we shall consider the experimental results of Broderius and Kahl (15) on the mortality of fathead minnows (*Pimephales promelas*) in the presence of each of 27 different organic chemicals. A plot of the results obtained by these authors is shown in Fig. 1, where the toxicities have been normalized (M 96h LC50) to the potency of 1-octanol. The normalized experimental results and the probit line ($\alpha = 59.1$, $\beta = 13.5$) are as reported by Broderius and Kahl. In addition, we have included a Weibull curve ($A = 53.16$, $\eta = 5.81$) fitted to the experimental points.

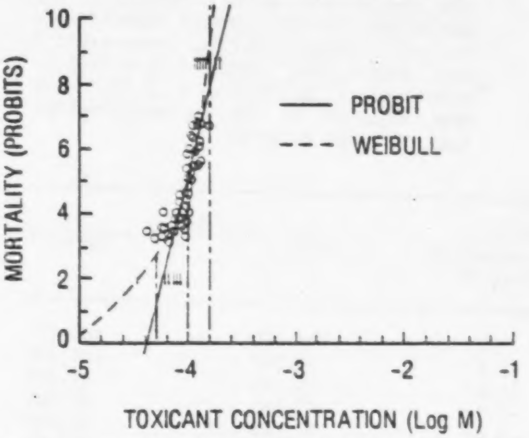

Fig. 1. Mortality vs. toxicant concentration for 27 different organic chemicals. The mortalities are normalized (96h LC50) to that of 1-octanol. Experimental points and the probit line are from Broderius and Kahl (15). In addition, we have included a Weibull function with parameters $A = 53.16$ and $\eta = 5.81$ that have been adjusted to fit the experimental data.

Because the normalization was made with respect to the LC50-values only, and not the slopes, it is not entirely appropriate to use a statistical criterion such as chi-square to compare the goodness of fit of the two models.

However, since the slopes of the 27 dose-response curves were fairly similar, and the mortalities were fairly evenly distributed between 0 and 100%, a comparison may still be valid. From Fig. 1 it is seen that the test data tend to follow the curved Weibull function rather than the straight probit line. Similar observations on other bioassay data were made previously (9).

The probit and Weibull models give comparable response rates for probit values between 4 and 6, but highly diverging values at the extremes. The mortalities from the Weibull function is the highest in both ends. This is important in the case where response functions obtained by fitting to intermediate test mortalities (e.g., between 10 and 90%) are used for extrapolation to high or low concentrations.

As may be seen from Table 3, the difference between the mortalities from the two models is rather trivial for values of log M between -4.1 and -3.9. However, at log M = -3.8, the probit model predicts that 255 out of 10^5 organisms will survive, while the corresponding number for the Weibull function is only 5. Similarly, at log M = -4.3, the probit model implies that almost no organisms are affected (only 4), whereas a total of 1230 are killed according to the Weibull model.

TABLE 3. Number of Fish Killed (fathead minnows, *Pimephales promelas*) out of an Initial Population of 10^5 as Predicted from the Probit and Weibull Models. The parameters of the probit model (α = 59.1; β = 13.5) are from Broderius and Kahl (15) , and those of the Weibull model (A = 53.16; η = 5.81) have been adjusted to fit the experimental data of these authors.

Toxicant Concentration (log M)	Model	
	Probit	Weibull
-3.6	100,000	100,000
-3.7	99,998	100,000
-3.8*	99,745	99,995
-3.9	92,645	92,681
-4.0*	53,983	49,634
-4.1	10,565	16,463
-4.2	466	4,608
-4.3*	4	1,230
-4.4	0	324
-4.5	0	85
-4.6	0	22
-4.7	0	6
-4.8	0	2
-4.9	0	0
-5.0	0	0

*Corresponding to broken vertical lines in Fig. 1

Algae. The models for one toxic substance listed in Table 2 have been applied to the growth of the diatom *Navicula incerta* exposed to Cd, Cu, Pb, and Zn. The raw data are from Rachlin, Jensen, and Warkentine (16).

The results for *Navicula incerta* are given in Tables 4 and Fig. 2. From Table 4 it is seen that the Weibull model provides the better fit compared to the probit and logit models when the number of degrees of freedom are two or more. The slope η appears to assume the value 0.5 for Cu, Pb, and Zn when growth rate is used as a parameter. The interpretation of η may be the number of toxicant molecules per receptor of the organisms, and the implication in the present case is, therefore, that each of the metals Cu, Pb, and Zn combines with two receptors.

A NONINTERACTIVE MULTIPLE TOXICITY MODEL

We have expanded Hewlett and Plackett's (17) bivariate normal model to include any monotone tolerance distribution for individual toxicants, such as a logit or Weibull distribution, and n toxicants (12). Let us consider a general bivariate model. Besides the parameters characterizing the individual dose-response curves (Table 2), there are two additional parameters: a similarity parameter λ and a correlation ρ of mortality tolerances (Group I organisms, e.g., fish) or cell division tolerances (Group III or IV organisms, e.g., algae).

The similarity parameter λ indicates whether the toxicants act on similar ($\lambda = 1$), different ($\lambda = 0$), or partially similar biological systems ($0 < \lambda < 1$). The other parameter ρ is a measure of the degree of correlation of the susceptibility of the organisms (Group I) to the two toxicants. For full correlation ($\rho = 1$), organisms that are very susceptible to one toxicant are also very susceptible to the other. In the case of full negative correlation ($\rho = -1$), there is an inverse relationship between the susceptibilities, e.g., organisms that are very susceptible to one toxicant are least affected by the other. Zero correlation ($\rho = 0$) means that there is no relationship between the susceptibilities of the organisms to the two toxicants, and all other values ($-1 < \rho < 1$) represent intermediate cases. For microorganisms, it is hypothesized that ρ should be one because all organisms are from the same clone and are in the same (Group III) or nearly the same (Group IV) physiological state.

The case of ($\lambda = 1$; $\rho = 1$) is characterized by the term concentration addition (C.A.), and the case of ($\lambda = 0$; $\rho = 0$) by the term response multiplication (R.M.). Computer programs to estimate the parameters of the univariate distributions in Table 2 are available (18). Also, the general noninteractive multiple toxicity model has been formulated into a computer program MULTOX which may be obtained from the same source (19).

Applications

Fish. We shall here analyze the results obtained by Anderson and Weber (20). They

TABLE 4. Fit of the Weibull, Probit, and Logit Distribution to Growth Data for the Diatom Navicula incerta. The Raw Data are from Rachlin, Jensen, and Warkentine (16). Concentrations are in mg/l.

Parameter	Toxicant	df*	Weibull A	Weibull n	Weibull χ^2	Probit α	Probit β	Probit χ^2	Logit θ	Logit ϕ	Logit χ^2
Growth Rate	Cd	1	-2.13	0.895 ±0.051	0.00031	3.71	1.53 ±0.03	0.000044	-2.18	1.12 ±0.004	3.1×10^{-8}
	Cu	2	-2.59	0.561 ±0.079	0.0019	3.49	0.831 ±0.127	0.0026	-2.61	0.641 ±0.093	0.0021
	Pb	3	-2.26	0.561 ±0.092	0.0071	3.74	0.807 ±0.154	0.0104	-2.19	0.625 ±0.116	0.0089
	Zn	6	-1.75	0.431 ±0.104	0.0345	4.01	0.665 ±0.166	0.0366	-1.66	0.495 ±0.123	0.0356
Yield	Cd	1	-1.31	0.797 ±0.088	0.0021	4.21	1.66 ±0.003	8.2×10^{-7}	-1.31	1.18 ±0.01	0.000020
	Cu	2	-1.72	0.554 ±0.084	0.0062	3.94	1.03 ±0.16	0.0084	-1.77	0.745 ±0.114	0.0075
	Pb	2	-2.07	0.650 ±0.098	0.0031	3.83	1.01 ±0.15	0.0040	-1.99	0.755 ±0.120	0.0038
	Zn	6	-2.59	0.958 ±0.109	0.0197	3.32	1.69 ±0.21	0.027	-2.91	1.27 ±0.15	0.0206

*Degrees of Freedom

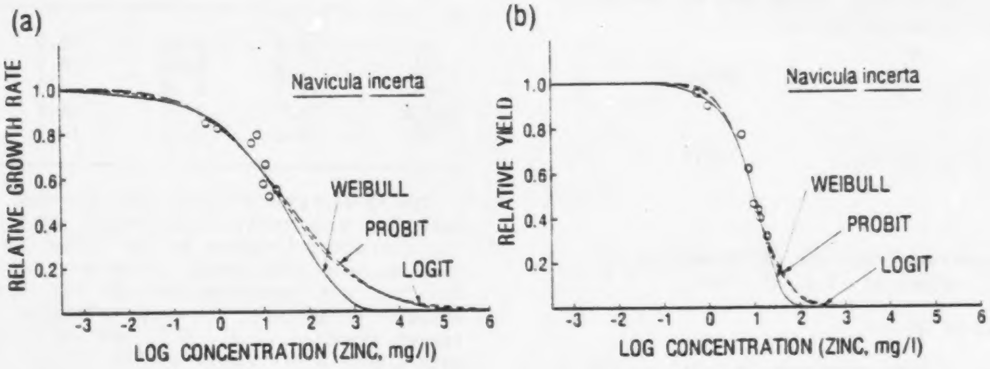

Fig. 2. Fit of the Weibull, probit and logit models to growth parameters for Navicula Incerta: (a) relative growth rate, and (b) relative yield, both based on cell number. The raw data are from Rachlin, Jensen, and Warkentine (16).

considered only R.M. and C.A. with parallel dose-response curves, i.e., identical β-values (Table 2), while we shall allow any correlation ρ between -1 and +1, partially similar systems, and C.A. with non-parallel dose-response curves. Also, in contrast to their approach, we include not only probit but also logit and Weibull transformations.

Basic probit lines for the action of the individual toxicants nickel (Ni), copper (Cu), potassium pentachlorophenate (PCP), dieldrin (HEOD), and potassium cyanide (CN) on male guppies (Poecilia reticulata) are given in Table 5. In this and the following tables, the weight of each lot of fish is the total weight of the ten fish in a batch. The weight W modifies the concentration M of a toxicant such that the "effective concentration" M/W^h (h = 0.67 - 0.81) remains the same either for a high actual concentration and high average weight of fish or a low actual concentration and a low average weight of fish. In other words, the important quantity is concentration per weight raised to the power h and not just concentration. Or: larger fish can tolerate higher concentrations for the same mortality rate.

TABLE 5. Probit of Mortality to Male Guppies (Poecilia reticulata) for Several Toxicants. M(mg/l) is the Concentration of the Toxicant and W(g) is the Weight of Each Lot of Fish. The relationships are from Anderson and Weber (20)

Toxicant	Probit of Mortality
Ni	$Y = -3.21 + 6.32 \log(M/W^{0.67})$
Cu	$Y = 11.4 + 7.46 \log(M/W^{0.72})$
PCP	$Y = 11.77 + 11.23 \log(M/W^{0.72})$
HEOD	$Y = 20.83 + 6.84 \log(M/W^{0.81})$
CN	$Y = 14.71 + 11.65 \log(M/W^{0.72})$

Logit and Weibull parameters corresponding to the probit parameters in Table 5 should preferably be derived from the original test data. However, since they were not available, we determined approximate parameters by a fitting process, using the weighting:

$$w_i = n_i \frac{Q_i}{1-Q_i} (\ln Q_i)^2 \qquad \text{Weibull}$$

$$w_i = n_i Q_i(1-Q_i) \qquad \text{logit}$$

where

Q_i = the survival fractions corresponding to N.E.D. values of -1.5, -1, -0.5, 1, 1.5

Y_i = probit of Q_i

n_i = number of test organisms in trial i (10) i = 1, 2, . . ., 7

The logit and Weibull parameters (Table 6) were then calculated using regressions based on the linear transformations in Table 2 and the weighting indicated above. As an example, the value of $M/W^{0.67}$ (Table 5) is calculated in the following manner, considering Ni at N.E.D. = 1.5:

$$6.5 = -3.21 + 6.32 \log\left(\frac{M}{W^{0.67}}\right)$$

or

$$\frac{W}{W^{0.67}} = 34.36$$

In the regression of the linear Weibull transformation $u = \ln k + \eta \ln z$, $z = z_1$ is then 34.36 and $u = u_1$ is given by $u_1 = \ln(-\ln(1-P)) = \ln(-\ln(0.067)) = 0.994$. Similar points are obtained for N.E.D. = 1.0, 0.5, 0, -0.5, -1.0, -1.5. The intercept A and slope η are then given by:

$$A = (1/D)[(\Sigma w_i u_i)(\Sigma w_i X_i^2) - (\Sigma w_i X_i)(w_i u_i X_i)]$$

$$\eta = (1/D)[(\Sigma w_i)(\Sigma w_i u_i X_i) - (\Sigma w_i X_i)(\Sigma w_i u_i)]$$

where $D = (\Sigma w_i)(\Sigma w_i X_i^2) - (\Sigma w_i X_i)^2$

$i = 1, \ldots, N; \quad N = 7$

$X_i = \ln z_i$

For N we obtain A = -9.4 and η = 2.99 (Table 6).

Table 6. Logit and Weibull Parameters Corresponding to the Probit Relationships of Table 5

Toxicant	Logit Parameters		Weibull Parameters	
	θ	ϕ	A	η
Ni	-13.9	4.66	-9.4	2.99
Cu	10.9	5.50	6.5	3.53
PCP	11.5	8.28	6.9	5.31
HEOD	26.9	5.04	16.8	3.24
CN	16.5	8.59	10.1	5.51

The bivariate fitting was carried out as indicated previously (12), except that we here use minimum chi-square as the criterion rather than maximum likelihood. However, because of the indirect determination of the logit and Weibull parameters, it was estimated that a larger stepsize, i.e., 0.1, was sufficient for both λ and ρ in search of the global minimum for χ^2 which is calculated according to the formula:

$$\chi^2 = \sum_{i=1}^{N} n_i \frac{(Q_i - q_i)^2}{Q_i(1-Q_i)}$$

where q_i = experimental survival fractions, e.g., 70% in the first case and 20% in the second (Table 7).

Q_i = calculated survival fractions.

n_i = number of test organisms in trial i (10).
N = number of trials (6).

We systematically calculate χ^2 for several combinations of λ and ρ. The pair producing the global minimum of χ^2 is retained.

The results for the binary mixtures (Ni, Cu), (PCP, HEOD), and (PCP, CN) are listed in Tables (7-9) and summarized in Table 10. There is little difference between the fits of the probit and logit models, both in terms of the optimum values of λ and ρ and the resulting χ^2. However, the Weibull model shows some distinctive differences. It produces the best fits for the (Ni, Cu) and (PCP, HEOD) pairs. For the (PCP, CN) pair the probit or logit models produce minimum χ^2 but this would appear to be less important because none of the fits are particularly good in that case ($P < 0.01$) The λ values are the same and the ρ values nearly so for a given binary mixture and different models (Table 10). The reason that the similarity parameter λ and the correlation ρ between the two tolerances are relatively insensitive to the form of the mathematical model here is that there are only ten fish in each experimental batch of the example (20). Thus, the models are essentially fitted to response probabilities between 10 and 90%, and in this range there is not a great deal of difference between the fits of the probit, logit, and Weibull models. However, as illustrated by Christensen and Chen (12), the situation is different when high or low response probabilities are included. In that case, not only will the estimates of λ and ρ depend upon the choice of model, but the probit model may not fit at all. The advantage of using non-normal bivariate tolerance models will, therefore, be particularly evident when extreme response probabilities are encountered as for example in models for carcinogenesis.

TABLE 7. Evaluation of the Joint Action of Ni and Cu on Male Guppies Based on the Parameters of Tables 5, 6 and the Computer Program MULTOX

Weight of Each Lot of Fish (g)	Concentrations (mg/l)		Calculated Percent Mortality for Min. Chi-Square			Observed Percent Mortality (20)
	Ni	Cu	Probit (λ=1; ρ=0.5)	Logit (λ=1; ρ=0.5)	Weibull (λ=1; ρ=0)	
1.53	12.23	0.049	16	16	19	30
1.07	15.56	0.082	83	83	88	80
1.27	14.17	0.084	66	67	68	70
1.30	10.77	0.063	28	28	29	30
1.23	15.15	0.071	64	65	66	80
1.23	14.79	0.058	53	53	53	80

TABLE 8. Evaluation of the Joint Action of PCP and HEOD on Male Guppies Based on the Parameters of Tables 5,6 and the Computer Program MULTOX

Weight of Each Lot of Fish (g)	Concentrations (mg/l)		Calculated Percent Mortality for Min. Chi-Square			Observed Percent Mortality (20)
	PCP	HEOD	Probit (λ=0.1; ρ=-0.1)	Logit (λ=0.1; ρ=0)	Weibull (λ=0.1; ρ=-0.2)	
1.51	0.26	0.005	30	28	34	10
2.15	0.40	0.00645	49	47	49	40
1.76	0.31	0.0063	45	42	46	50
1.79	0.40	0.0063	75	74	73	60
1.51	0.29	0.0048	39	37	42	70
1.94	0.41	0.0069	72	70	69	80

TABLE 9. Evaluation of the Joint Action of PCP and CN on Male Guppies Based on the Parameters of Tables 5,6 and the Computer Program MULTOX. The Weight of Each Lot of Fish Has Been Set to 1.50g

Concentrations (mg/l)		Calculated Percent Mortality for Min. Chi-Square			Observed Percent Mortality (20)
PCP	CN	Probit (λ=0.2; ρ=-0.8)	Logit (λ=0.2; ρ=-0.8)	Weibull (λ=0.1; ρ=-0.8)	
0.233	0.135	8.5	9.8	17	10
0.231	0.146	13	14	22	10
0.257	0.175	54	53	53	40
0.257	0.169	48	47	49	100
0.246	0.153	22	23	30	10
0.177	0.139	4.6	5.6	11	10

TABLE 10. Chi-Square for Binary Mixtures of Toxicants Considered in Tables 7-9 (four degrees of freedom).

Toxicants	Model		
	Probit	Logit	Weibull
Ni-Cu	5.79 (λ=1, ρ=0.5)	5.72 (λ=1; ρ=0.5)	5.38 (λ=1; ρ=0)
PCP-HEOD	7.83 (λ=0.1; ρ=-0.1)	8.12 (λ=0.1; ρ=0	7.72 (λ=0.1; ρ=-0.2)
PCP-CN	13.2 (λ=0.2; ρ=-0.8)	13.2 (λ=0.2; ρ=-0.7)	14.2 (λ=0.2; ρ=-0.8)

The estimation of parameters when three or more toxicants are considered, using the above method with χ^2 as criterion, is very cumbersome and we have not attempted it. Other means of estimating parameters are currently being explored.

Isobolograms for the three binary mixtures of Table 10, based on the Weibull model, are shown in Figure 3. The curves are drawn for three values of the non-response probability Q: 0.1, 0.5, and 0.9. The symbols M and W of the modified concentration are defined in Table 5, and h (0.67-00.81) is the exponent of the weight of each lot of fish. C_1 and C_2 are the values of M/W**h for each toxicant that will give the desired response when acting separately. It is clear that the isoboles for Cu and Ni (Figure 3a) are close to defining a straight-line relationship characteristic of C.A. This might be expected since $\lambda = 1$; and although $\rho = 0$, the variation of the response for ρ between 0 and 1 is modest (12). The isoboles for HEOD and PCP (Figure 3b) are typical when $\eta > 1$ for R.M. which is indicated by the values of λ and ρ that are both close to zero. Except for an interchange of indices, these curves are in fact similar to the curve labelled 1 in Figure 3a of ref. (12), which is strictly valid for R.M.

Algae. The use of the above multiple toxicity model for algal growth rate based on cell number was considered previously (21, 22). The growth rate of the green alga Selenastrum capricornutum and the blue-green Synechococcus leopoliensis was modeled as a function of ionic concentrations of Ni and Zn.

We consider here the growth rate of Selenastrum based on cell volume. The experiment was designed such that for each point, equitoxic concentrations of Ni^{2+} and Zn^{2+} would be combined. The culturing methods were as described by the U.S. Environmental Protection Agency (23), and ionic concentrations were calculated by the equilibrium speciation program MINEQL (24). The results of such an experiment are shown in Fig. 4. Just as for growth rate based on cell number (21), the joint action appears to be close to C.A. (ρ =1; λ = 0.9). However, here the best model is logit rather than Weibull.

CONCLUSIONS

The following conclusions may be drawn from the present study:

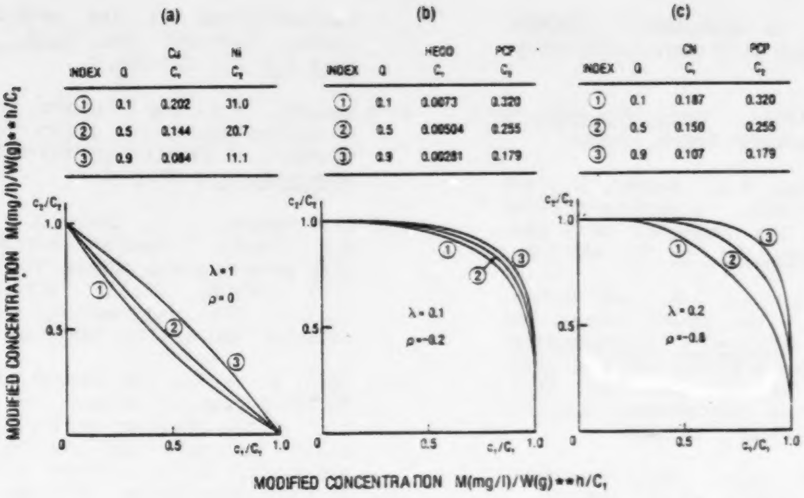

Fig. 3. Isobolograms for the effect of (a) (Ni, Cu), (b) (PCP, HEOD), and (c) (PCP, CN) on male guppies based on the Weibull model with optimum values of λ and ρ (Table 10).

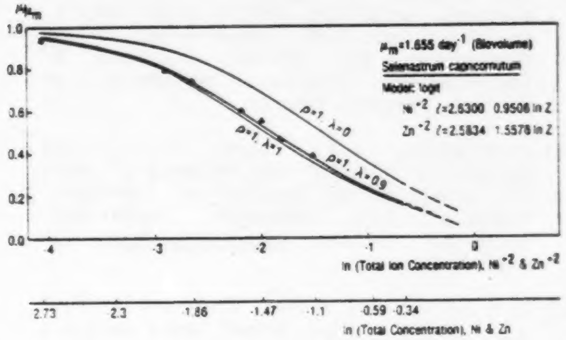

Fig. 4. Combined effect of Ni^{2+} and Zn^{2+} on the growth rate based on cell volume (biomass) of the green alga Selenastrum capricornutum. The test results are best fitted by a bivariate logit model with $\lambda = 0.9$ and $\rho = 1$.

(1) The Weibull model should be given serious consideration as a replacement for the probit model as a general dose-response function for the quantal response of macroorganisms with a tolerance distribution (Group I organisms). The main reason is that the Weibull model appears to give a better fit to experimental data, and that it, therefore, is more likely to provide valid mortality estimates by extrapolation, particularly to low concentrations. The better fit of this model supports a previously suggested mechanistic-probabilistic basis in terms of chemical reactions between toxicant molecules and a key receptor of the organism.

(2) Literature data for the growth rate and yield of the diatom Navicula incerta inhibited by Cd, Cu, Pb, or Zn were fitted to the univariate Weibull, probit, and logit models. The Weibull model provides generally the best fit, thus supporting a basis which was previously developed for microorganisms (Group III and IV organisms) when the growth rate based on cell number was modeled as a function of toxicant concentration.

(3) A general noninteractive multiple toxicity model was applied to literature data for the toxicity of binary mixtures of Ni, Cu, PCP, HEOD, and CN to male guppies (Poecilia reticulata). We confirm that the action of (Ni, Cu) and (PCP, HEOD) indeed may be approximately characterized by C.A. and R.M., respectively. The estimates of the similarity parameter λ and the correlation coefficient ρ are relatively insensitive to the choice of model here because the response probabilities mainly are in the range between 10 and 90%, and in this range there is not much difference between the fits provided by the three models. Nevertheless, in both of the above cases, the Weibull model gives minimum chi-square.

(4) The combined effects of Ni^{2+} and Zn^{2+} on the growth rate based on cell volume (biomass) of the green alga Selenastrum capricornutum were approximately according to C.A., with $\lambda = 0.9$ and $\rho = 1$. While previous bioassays, in which the growth rate was based on cell number, demonstrated that the Weibull model was preferable, the present results, based on cell volume, indicate that the logit model is best suited to describe the combined response.

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DISCUSSION

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Christensen presents examples of the application of a noninteraction multiple toxicant model to several data sets, including mortality of fathead minnows and population growth rate and yield of diatoms. He concludes that the mixture Weibull is a preferred model for similar acting chemical pairs and infers the type of noninteraction between the mixture components by the values of the model's parameter estimates. There are three aspects of this work that should receive critical attention: the usefulness of the mixture models as descriptors of mixture toxicity, the biological interpretation of the Weibull and its parameters, and the future of modeling binary mixtures.

The feeling one gets in reading the paper is that the results of the mixture models are tantalizing yet incomplete. To his credit, Christensen's work does include many desirable characteristics: multiple dose levels, different types of toxicants, two very different species, and well-defined biological end points. But several items are missing: the models are not presented, the dose adjustment model (divide dose by a power of body weight) has no statistics on its parameters that might suggest the validity of such an adjustment, and the descriptions of the model fits do not include significance levels or even graphs. The latter is important since the information that is provided (chi-square values) shows only a marginally better fit for the Weibull, which is an inadequate criterion for model preference.

Of more concern, perhaps, is the motivation for the models. Christensen states that the models are to be considered empirical, yet he then infers biological meaning to the value of the Weibull parameters. The biological properties should have been established first (e.g., Cu and Ni are toxicologically similar) and then shown to be consistent with the model's results (e.g., lambda=1). Two similarly acting toxicants are often characterized as being dilutions or concentrations of one another so that, once adjusted for potency differences, the two chemicals should have the same dose-response curves. Because of this, is seems that two similar chemicals (lambda=1) should also have the same tolerance distributions (rho=1). The inclusion of this constraint, and verification by actual data, would improve the support for Christensen's approach. Without such support, inferences about toxic similarity from parameter values are not believable.

The use of mortality as the toxicity indicator raises several issues. First, mortality is usually interpreted as a non-specific toxic end point, and thus it provides little information on toxic mechanism. Consequently, the inference about toxic similarity is confusing. The usual definition of toxic similarity (EPA, 1986) is that the same tissues and organs are affected, and that the same type of damage or lesion results. In contrast, mortality usually results from failure of several organs and the exact cause of death is rarely identified. Second, mortality is useful primarily for assessment of ecosystems. Presence/absence and population size of indicator organisms have been used successfully for years to evaluate water quality of lakes and streams. But mortality is not particularly helpful for human risk assessment. Particularly for systemic toxicants (chemicals with a toxic threshold), the preferred data would include doses showing several degrees of sublethal effects along with doses showing no effects.

The problems with developing a general mixture assessment methodology are only touched on in Christensen's discussion. These include having more than two components in the mixture, multiple end points and varying degrees of severity for each end point. The extension of binary models, as has been done for multistage cancer models (Thorslund and Charnley, 1986), is one approach for evaluating several components, particularly for single end points. But the general n-chemical model can become intractable, as Christensen mentions, even for one end point. The extension to multiple end points by traditional methods seems out of the question.

One useful approach we are investigating is to combine expert judgment with generalized linear models. We have adapted the work of McCullagh (McCullagh and Nelder, 1983) to give a multi-chemical model which uses judgments of the overall severity of the toxic reponse in lieu of response rates or numerical intensity measures of specific effects. In this way, data describing several end points, even purely qualitative descriptions, can be modeled to give estimates of an "acceptable" dose or of a dose corresponding to a low risk. Consider the following data for dieldrin-induced nephritis (Fitzhugh et al., 1964):

DOSE (ppm)	LESIONS None	Slight	Moderate	Severe
0.0	5	5	6	1
0.5	5	9	5	3
2	9	8	6	0
10	5	6	6	1
50	5	6	6	3
100	5	3	2	8
150	1	2	7	1

The multiple response curves plotted against dose (given in Fig. 1) are not easily interpreted in terms of overall risk. The cumulative response (Fig. 2) separates the severity groups and allows an estimation of the probability of seeing a given severity or less for any given dose. The statistical approach we are developing is similar. The steps are as follows:

1. The main covariables (dose, duration, species, route) are represented by categories (intervals for the continuous variables).

2. The response is coded in terms of a toxicologist's judgment of overall severity to the animal. This code will be from a predefined set of categories.

3. Apply McCullagh's approach for ordered categorical data:

    a. Identify a link function to transform the original response variable into one that is linear in the covariables. We are investigating the log cumulative odds:

    $$q_{ij} = \ln(c_{ij}/(1-c_{ij})),$$

    where:

    $$c_{ij} = \sum_{k=1}^{j} p_{ij}$$

    For a single covariate, say dose, then j indexes severity, i indexes dose, and q_{ij} is the log odds of the severity being in category j or less, given a dose in category i. Here p_{ik} is the fraction of responses of severity k at dose i.

    b. Regress q on the covariates: $q = Ax + b$

    c. Calculate the risk of response from the link function. For a dose d, and severity s:

    $$r_{ds} = \Pr\,[\text{response at level } s \text{ or less, given dose } d]$$

    $$= \exp(Ad+b)/(1 + \exp(Ad+b))$$

The primary advantage of this method is that the data constraints are minimal; virtually any type of toxicity data can be modeled to give doses that are "acceptable" or that correspond to low risk. In addition, this approach yields maximum likelihood estimates. The disadvantages are that little indication is given of the mechanisms of toxicity, and that the dose-response relation is limited by the precision of the dose and response categories. What remains to be checked is the numerical performance of this method, and the ease of determining a suitable link function. Note that this approach also works with complex (say, for n > 20 chemicals) mixtures. If the mixture is relatively stable over time, then it can be treated as a singlechemical entity and the severity judgment reflects the impact on the test animal of all effects from all components.

In summary, Christensen's work appears most applicable to ecosystem assessment of simple mixtures. The use of Weibull parameters to indicate the nature of the interactions is intriguing, and should be pursued, including validation by chemical pairs with known mechanisms of toxic interaction. Further, one must agree with his caution against the habitual preference for the probit model. For human risk assessment, however, it seems that other approaches such as I have outlined will be required, particularly those which place fewer demands on the quality and quantity of the data.

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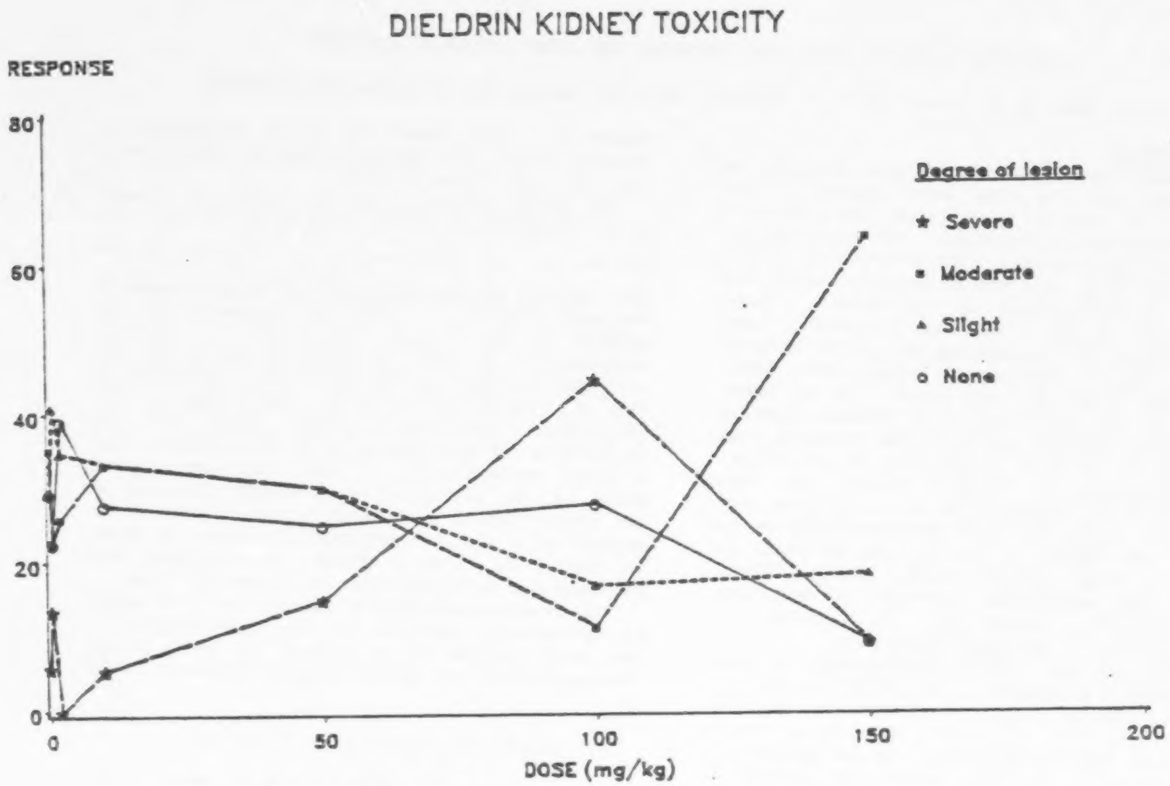

FIGURE 1. EXAMPLE OF DIFFICULT INTERPRETATION OF STANDARD OVERLAID DOSE-RESPONSE PLOTS OF MULTIPLE EFFECTS. SOURCE: FITZHUGH ET AL., 1964.

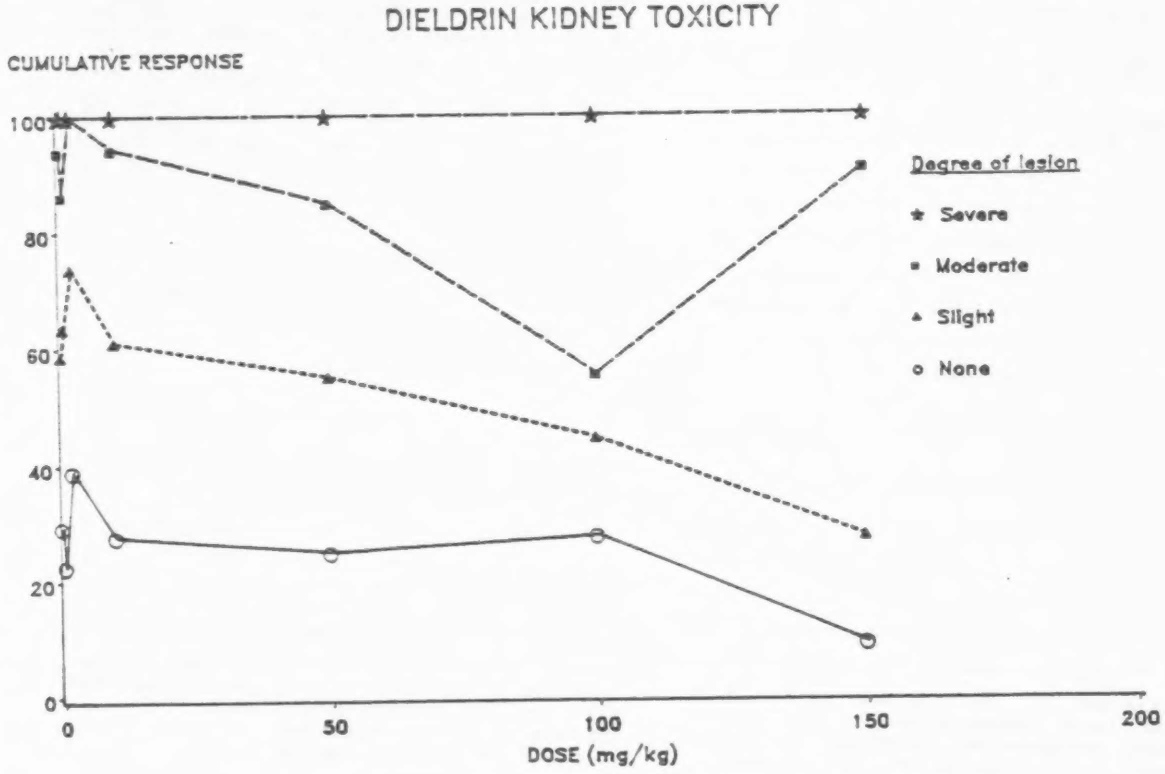

FIGURE 2. EXAMPLE OF IMPROVED INTERPRETATION DUE TO SEPARATION OF CURVES BY USING CUMULATIVE RESPONSE. SOURCE: FITZHUGH ET AL., 1964.

A RESPONSE-ADDITIVE MODEL FOR ASSESSING THE JOINT ACTION OF MIXTURES

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1. INTRODUCTION

Individuals are exposed to various mixtures of toxic chemicals in the environment. The assessment of health risks from the exposure becomes increasingly important. The construction of mathematical models for predicting joint toxicity by using only the information about the toxicity of individuals is difficult. Dose-addition and response addition frequently have been mentioned for evaluating the joint effects of two toxicants, (Shelton and Weber, 1981; Reif, 1984). Two chemicals are said to be dose-additive or are said to have "simple similar joint action" (Finney, 1971) if one chemical acts exactly as if it were a dilution of the other. Response-addition or effect-addition has been used in different contexts in the literature; the most common definition for response-addition is that combined effect of the mixture is equal to the sum of each effect alone, (Reif, 1984). Synergism and antagonism represent a deviation from additivity under the null model of dose-additivity or response-additivity.

Hamilton and Hoel (1980) distinguished between two purposes for studying the joint actions of chemicals, "those studies conducted to provide risk estimates from the joint exposure and those studies conducted to elucidate the mechanisms of joint toxicity." In this paper, we propose a mathematical model for presenting and analyzing the data from mixture studies. The dose-response function is modeled as a function of both the proportions of chemicals in the mixture and the total concentration of the chemicals. A response-additivity is introduced for assessing the joint action of chemicals.

2. RESPONSE-ADDITIVE MODEL

Let x_i be the proportion of chemical C_i $(i=1,2)$ in a mixture with total concentration T. Then $t_i=Tx_i$ represents the concentration of chemical C_i in the mixture. Suppose that $F_i(t_i)$ represents the (dose) response function of chemical C_i at a dose level t_i. It is assumed that the response of the mixture, $R(t_1,t_2) = R(x_1,x_2,T)$, can be expressed as

$$R(x_1,x_2,T) = x_1 F_1(T) + x_2 F_2(T) + E(x_1,x_2,T). \quad (1)$$

The terms $x_1F_1(T)$ and $x_2F_2(T)$ may represent the "expected" responses produced by administration of the single chemicals, and $E(x_1,x_2,T)$ then represents the "excess" of the response over $x_1F_1(T) + x_2F_2(T)$ produced by the mixture.

In equation (1), the data were collected at different total concentrations of the mixture with each concentration consisting of several different proportions of the two chemicals, Figure 1. This model was first introduced by Scheffe (1958) for studying mixture experiments with only one concentration.

The joint action of the two chemicals is said to be "response-additive" if $E(x_1,x_2,T) = 0$, for all x_1, x_2, and T with $x_1+x_2=1$. That is, if the joint action of two chemicals can be predicted by response-additivity, then the response of the mixture at the (total) concentration T can be represented by the weighted average of the responses produced by the individual chemicals at the concentration T with the weights for individual responses being equal to the proportions of the chemicals in the mixture. For a fixed concentration T, the response-additive model can be expressed as a linear function of the proportion of a chemical in the mixture. An example of a plot of response-additivity is shown in Figure 2. For a fixed concentration the response can be represented by a straight line.

The response-additivity defined in this paper is conceptually parallel to the dose-additivity. The joint action of a mixture is said to be "dose-additive" or simple similar (Finney, 1971) if

$$\begin{aligned} R(x_1,x_2,T) &= F_1(x_1T+mx_2T) = F_1(t_1+mt_2) \\ &= F_2(x_1T/m+x_2T) = F_2(t_1/m+t_2) \quad (2) \end{aligned}$$

where m represents the relative potency of the second chemical to the first. (A more general form of dose-additivity allows m to be a function of T.) A common method to present the dose-additivity is to use the isobolographic analysis which shows the various combinations of dose levels of the two chemicals which produce the same level of response. The isobologram for dose-additivity can be represented by a set of straight lines. An example of a plot of the isoboles for dose-additivity is shown in Figure 3. The isobole of a given response is a straight line.

Without loss of generality, assume that $F_2(T) > F_1(T)$, i.e. $m>1$. If the joint action of C_1 and C_2 is dose-additive then

$$\begin{aligned} &F_2(T) > F_2[(x_1/m+x_2)T] = \\ &\qquad F_1[(x_1+mx_2)T] > F_1(T) \quad (3) \end{aligned}$$

That is, the response predicted by dose-additivity is bounded by the two responses produced by the single chemicals of the same total concentration. If the response function F_1 is convex in the (dose) interval (T,mT), then the response predicted by dose-additivity is less than that predicted by response-additivity. On the other hand, if the response function F_1 is concave in the interval (T,mT), then response predicted by dose-additivity is greater than that predicted by response-additivity, Figure 4. Therefore, a definition for "additive" joint action of two chemicals can be

$$F_2(T) > R(x_1,x_2,T) > F_1(T) \qquad (4)$$

for any x_1,x_2. and T with $x_1 + x_2 = 1$. Two non-additive actions, synergism and antagonism, can be defined by using equation (4). The synergistic (antagonistic) action occurs if the response of the mixture is greater (less) than the additive response, that is,

$$R(x_1,x_2,T) > F_2(T)[R(x_1,x_2,T) < F_1(T)] \qquad (5)$$

This definition agrees with that of Vendetti and Goldin (1964) for studying the combination of two drugs.

3. ASSESSMENT OF INTERACTIVE ACTION

Suppose that the purpose of the experiment is to understand the underlying joint toxicity (interaction) of chemical combinations. Terminologies used for describing the joint actions of mixtures are interaction, independence, synergism, antagonism, and additivity. Unfortunately, these terms mean different things to different authors (Kodell and Pounds, 1985). Equations (4) and (5) define three possible models for characterizing the joint action of two chemicals. The assumption for the response-additive model defined in this paper is that the sites of primary action of the two chemicals are the same; this type of action is called similar joint action according to the classification of Plackett and Hewlett (1967). The joint action of two chemicals is simple similar or noninteractive if the presence of one chemical does influence the action of the other. Dose-additivity commonly has been used for assessing the interactive effects between two drugs in pharmacology. In this section, we apply the response-additive model to assess dose-additive joint action.

Suppose chemical C_2 is m times more potent than C_1 at dose T. To assess dose-additive, the dose measurement for chemical C_1 is scaled as $T' = mT$ so that both chemicals are equipotent, i.e., $F_1(T') = F_2(T')$. At the "concentration T'" in the mixture, the response predicted by response-additivity, Equation (1) is

$$R(x_1,x_2,T') = x_1F_1(T') + x_2F_2(T') = F_1(mT)$$

for any "proportions" x_1 and x_2. The response predicted by dose-additivity, Equation (2) is

$$R(x_1,x_2,T') = F_1(x_1T'+mx_2T') = F_1(mT) .$$

That is, at the concentration T' the response of the mixture predicted by dose-additivity and response-additivity is constant regardless of the proportions of individual chemicals in the mixture.

A procedure for testing dose-additivity can be constructed. Suppose that doses mT of C_1 and dosage T of C_2 produce the same level, p, of response, e.g., 50% effect. (This can be obtained by plotting the dose-response curves of each chemical.) The concentrations mT of C_1 and T of C_2 will be used as the standard preparations for constituting various mixtures of the experiment. Each mixture will contain x_1mT of compound C_1 and x_2T of chemical C_2, where $x_1,x_2 > 0$, and $x_1+x_2=1$. Let n_j denote the number of subjects in the experiment and r_j denote the observed number of effects in the j-th preparation (mixture). The hypothesis of dose-additivity can be test by using the chi-square test for homogeneity

$$X = \sum_{j=1}^{g} \frac{(r_j - n_j\,p)^2}{n_j\,p\,(1-p)}$$

where g is the number of preparatinos. If the two chemicals are dose additive, then X has a chi-square distribution with g degrees of freedom.

4. RESPONSE SURFACE ANALYSIS

Suppose that the purpose of an experiment is to study the relation between the different dose combinations with the responses. The experimenter may be interested in finding a suitable approximating function for the purpose of predicting future responses over a range of dosage, or determining what dose combinations (if any) can yield an optimum as far as the response concerned. The common approach of this problem is by a statistical curve fitting technique or a so-called response surface method.

Assume that the observed values y from the mixture contain variations e, the mixture responses can be written as

$$y = R(x_1,x_2,T) + e \qquad (6)$$

The variations e are assumed to be independently and normally distributed with zero mean and common variance. The functions $F_i(T)$ and $E(x_1,x_2,T)$, in general, can be represented by polynomial forms; that is, equation (1) can be expressed as

$$\begin{aligned} R(x_1,x_2,T) = {} & x_1 \left(\textstyle\sum_{k=0} b_1{}^k T^k\right) + x_2 \left(\textstyle\sum_{k=0} b_2{}^k T^k\right) \\ & + x_1\,x_2 \left(\textstyle\sum_{k=1} b_{12}{}^k\,T^k\right) + x_1^2\,x_2 \left(\textstyle\sum_{k=0} b_{112}{}^k\,T^k\right) \\ & + \ldots \end{aligned} \qquad (7)$$

For practical purposes, lower-degree polynomials are normally fitted. For example, a quadratic response model, a second degree polynomial function for x and T, for the mixture can be expressed as

$$\begin{aligned} R(x_1,x_2,T) &= \\ &x_1(b_1{}^0+b_1{}^1T+b_1{}^2T^2) + x_2(b_2{}^0+b_2{}^1T+b_2{}^2T^2) \\ &+ x_1x_2(b_{12}{}^0+b_{12}{}^1T+b_{12}{}^2T^2) \\ = \quad &x_1B_1(T) + x_2B_2(T) + x_1x_2B_{12}(T) \qquad (8) \end{aligned}$$

where $B_1(T)$, $B_2(T)$, and $B_{12}(T)$ are defined by the last equality of the equation. This model was proposed by Piepel and Cornell (1985), and was referred to as the mixture-amount model. Non-polynomial functions of dose T, e.g., log T, may be appropriate for certain bioassay responses.

It can be shown that the maximum response occurs in the experimental dose range if $B_{12}(T)>0$ provided that $B_1(T) + B_{12}(T) > B_2(T)$ and $B_2(T) + B_{12}(T) > B_1(T)$ for all T; similarly, the minimum response occurs in the experimental dose range if $B_{12}(T) < 0$ provided that $B_1(T) + B_{12}(T) < B_2(T)$ and $B_2(T) + B_{12}(T) < B_1(T)$.

Equation (7), alternately, can be expressed as

$$\begin{aligned} R(x_1,x_2;T) = &(b_1{}^0x_1 + b_2{}^0x_2 + b_{12}{}^0x_1x_2) \\ &+ (b_1{}^1x_1 + b_2{}^1x_2 + b_{12}{}^1x_1x_2)\,T \\ &+ (b_1{}^2x_1 + b_2{}^2x_2 + b_{12}{}^2x_1x_2)\,T^2 \quad . \end{aligned} \qquad (9)$$

When the experimental dose levels are coded to have zero mean (e.g., -1,0,1 for three concentrations), the coefficients have interpretations (Piepel and Cornell, 1985), e.g., the intercept term $(b_1{}^0x_1 + b_2{}^0x_2 + b_{12}{}^0x_1x_2)$ represent linear and nonlinear effects of the proportions in the mixture at the average concentration of the experiment.

If $b_{12}{}^0 = b_{12}{}^1 = b_{12}{}^2 = 0$, then the joint action of the two chemicals is response-additive; the response is linear with the proportion of a given chemical at each concentration (Figure 2). Three special situations are of interest:

1) If $b_1{}^1 = b_2{}^1 = b_1{}^2 = b_2{}^2 = 0$, then $R(x_1,x_2;T) = (b_1{}^0x_1 + b_2{}^0x_2)$; the lines in Figure 2 are coincident, the chemical concentration has no effect on the response.

2) If $b_1{}^1 = b_2{}^1$ and $b_1{}^2 = b_2{}^2$, then $R(x_1,x_2;T) = (b_1{}^0x_1 + b_2{}^0x_2) + b_1{}^1T + b_1{}^2T^2$; the lines in Figure 2 are parallel, the response increases by a constant amount as concentration increases.

3) If $b_1{}^0 = b_2{}^0 = b_1{}^1 = b_2{}^1 = 0$, then $R(x_1,x_2;T) = (b_1{}^1x_1 + b_2{}^1x_1 + b_2{}^1x_2)T$; the lines in Figure 2 are not parallel, the response increases proportionally with the concentration.

Equation (9) assumes that the experimental variations are normally independently distributed with zero mean and common variance. However, many data collected from the bioassay experiments do not follow the model assumptions. For example, Snee and Irr (1981) found that mutagenesis data collected from a mammalian cell assay system did not satisfy the assumptions of normality and constant variance. Various transformations can be used to achieve the model assumptions. For analyzing dose-response relationships of mutagenesis data, Snee and Irr (1981) suggested using the Box-Cox (1964) power transformation model

$$\begin{aligned} y^{\lambda} &= [R(x_1,x_2)]^{\lambda} + e \quad \text{for} \quad \lambda \neq 0; \qquad (10) \\ \log y &= \log\,[R(x_1,x_2)] + e \quad \text{for} \quad \lambda = 0 \;, \end{aligned}$$

where λ is the power transformation parameter to be estimated from the data. An application of the model is given in the next section.

5. EXAMPLE

An experiment was conducted to study the effects of mixtures of 1-nitrobenzo(a)pyrene (1-NBP) and 3-NBP on mutation induction in the Salmonella reversion assay. Both chemicals are suspected environmental contaminants and are potent direct-acting mutagens in Salmonella without exogenous activation (Pitts et al., 1984; Chou et al., 1984). Assays were performed with Salmonella typhimurium tester strain TA98 in the absence of exogenous metabolic activation using the methods described in Maron and Ames (1983). 1-NBP and 3-NBP were synthesized, free from contaminating isomers, by the methods of Chou et al., 1984). Mixtures of the two chemicals were prepared using seven different proportions of the two mutagens at the fixed total concentrations of 0.1, 0.2, and 0.4 ug of mutagen per plate. The mixture proportions and the experimental results are shown in Table 1.

TABLE 1. The number of mutants per plate produced by mixtures of 1-NBP and 3-NBP

1-NBP:3-NBP Ratio	Revertants per Plate 0.1	0.2	0.4
1:0	150,171,151	212,213,183	216,198,237
4:1	219,165,196	258,333,349	339,328,305
2:1	204,197,208	462,393,418	604,520,490
1:1	206,202,196	480,495,475	660,621,572
1:2	213,237,205	379,418,389	612,737,491
1:4	255,284,275	527,503,489	471,660,605
0:1	194,176,210	286,264,289	315,333,305

The Box-Cox power transformation was used to ensure that the assumptions of normality and homogeneous variance of experimental error were satisfied. Using the method given by Irr and Snee (1982) to calculate the power parameter λ; the estimated value of λ was approximately 0.20. Thus, the transformation $y^{0.2}$ was used to fit the dose-response functions for subsequent analyses. The fitted equation with the estimated coefficient standard errors for the data from Table 1 is

$$\begin{aligned} y = {} & (\underset{(12.0)}{183}\ \text{1-NBP} + \underset{(16.7)}{283}\ \text{3-NBP} + \underset{(73.1)}{760}\ \text{1-NBP*3-NBP}) \\ & + (\underset{(99.0)}{147}\ \text{1-NBP} + \underset{(139.4)}{435}\ \text{3-NBP} + \underset{(610.9)}{4190}\ \text{1-NBP*3-NBP})T \end{aligned} \tag{11}$$

where T is coded as -.0133, -0.033, and 0.166. Note that the coefficients for the quadratic function of T are not significant. Equation (11) shows mutagenic responses on 1-NBP and 3-NBP, and the responses produced by each chemical are not equal. The effect of the total concentration of the mixture is linear with the response. Increasing the total concentration affects both the linear terms, $b_1{}^1 = 147$ and $b_2{}^1 = 435$, and the nonlinear term, $b_{12}{}^1 = 4190$, in the mixture components. Moreover, it can be shown that a synergistic joint action between the two chemicals in the experimental dose range, total concentration from 0.1 and 0.4 ug/ml, and the mixture with proportions of 1-NBP to 3-NBP about 0.43 to 0.57 at total concentration 0.4 ug/ml can produce the strongest mutagenic effect.

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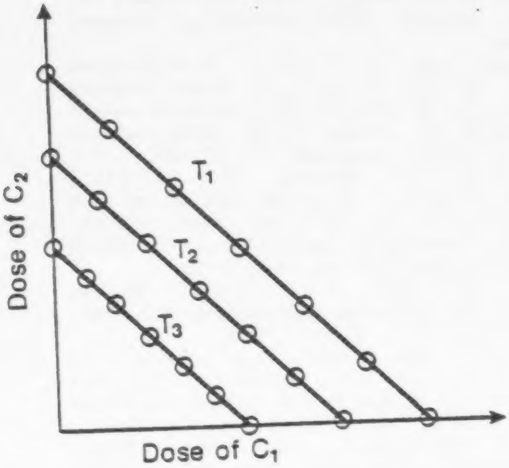
FIGURE 1. Mixture Design



T1
T2
T3
Dose of C2
Dose of C1

FIGURE 2. Response-Additivity

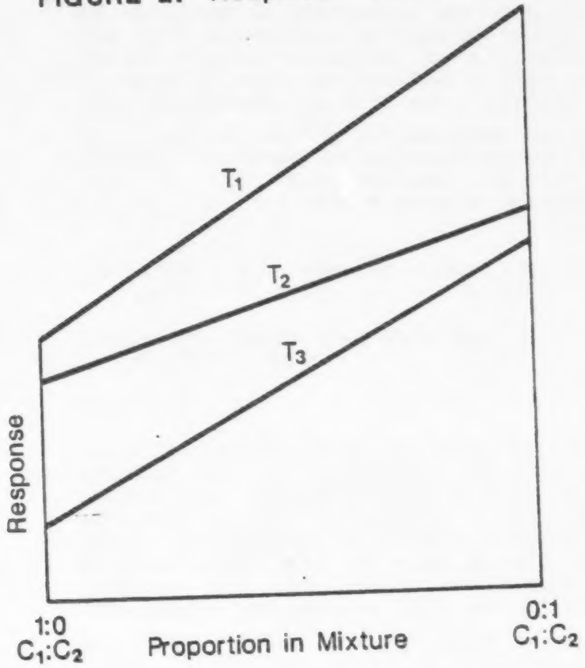
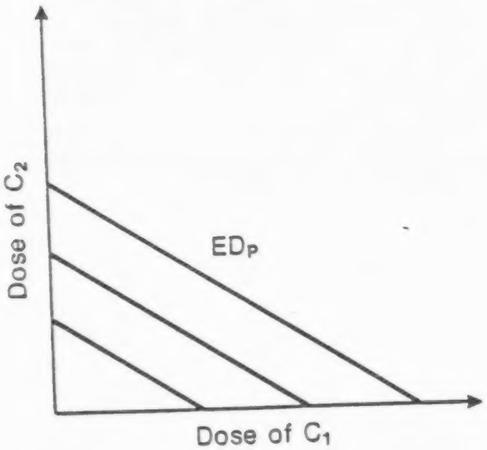
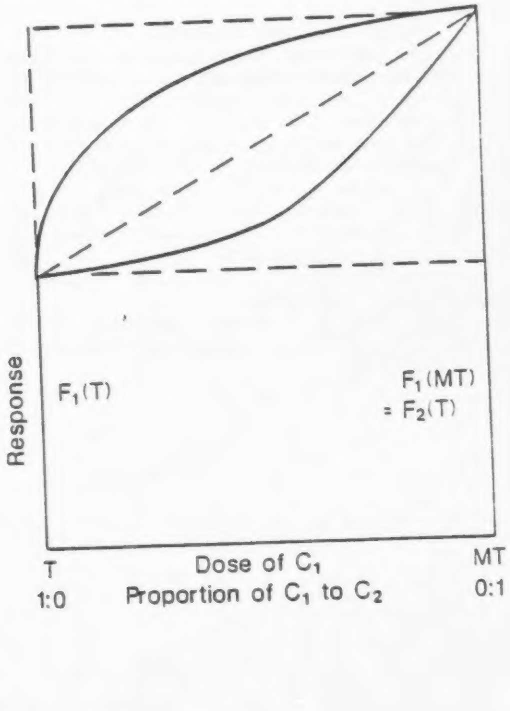


FIGURE 3. Dose-Additivity



EDP
Dose of C2
Dose of C1

FIGURE 4. Response from Dose-Additivity



DISCUSSION

Elizabeth H. Margosches, U. S. Environmental Protection Agency

I am pleased to have the opportunity to comment on this paper. When Dr. Chen first sent me the paper, several directions for comments came to mind. At this conference we have already heard many speakers refer to the properties of response additivity. Nevertheless, there are some special points here.

Chen *et al.* define a response additive model for which the outcome is the same as that under the dose additive assumption, and then proposes to test for dose additivity. This special case, however, where $E(x_1,x_2,T) = 0$, is, as Dr. Kodell pointed out yesterday, the exponential case, which has many well defined properties. One of the problems here is that the method works from a count of effects. Among the pluses: a test statistic is proposed; data are used on a noncancer endpoint, mutagenicity.

Chen *et al.* quote Hamilton and Hoel (1980) regarding two purposes for studying the joint actions of chemicals, as shown in Table 1. Studies may be conducted to provide risk estimates from joint exposure and they may be conducted to elucidate mechanisms of joint toxicity. The emphasis in Chen *et al.* appears to be on the latter and, as we've heard from several speakers, this is an important facet of research. As an EPA statistician, however, I must admit my concern is more with the former, although our interest is in both foci.

Can we expect one study to assist us in both endeavors? Probably not. Can we find one method of modeling to help in both?

What are the modeling questions asked in these two perspectives? In the first, we assume the components are unknown. We then try to predict the curve at some other dose than that studied. In the second, we can assume the components are known. Then we try to decide if, at some dose, there is joint action (or compounded effect). Any model that is chosen for use can only reflect the extent of joint activity built into it. Similarly, the shapes at low doses, the thresholds, etc., depend on the underlying postulates, not necessarily the true state of nature.

Thus, the two perspectives must have different analyses. Providing risk estimates from joint exposure calls for procedures that are robust against misspecification in the range of interest. Elucidating mechanisms calls for tests of full *versus* reduced models like those of which Dr. Machado spoke earlier. Chen *et al.* have provided conditions for maximum response and minimum response in the experimental range. What about in the low dose range where I have to work so often? Can the cancer model of which Dr. Thorslund spoke earlier help with transformed cell assay data?

But it seems one of the greatest limits we have placed on ourselves so far is that of dealing with substances in pairs. As Dr. Litt described yesterday, the Agency must deal on a daily basis with toxicants combined in both unidentified and unquantified mixtures, e.g., pesticides, waste dumps. We need methodology to take us beyond pairs.

What are our barriers to extension? I won't pretend to have identified all of these, and I offer just a few thoughts on ways statisticians have already extended themselves in other settings. Three that come to mind have entered into several papers at this symposium. (1)Looking at all the cross-products: this becomes quite cumbersome with more than two compounds in anything beyond near linear responses. Let's consider adopting a matrix notation, so useful in the analogous leap in regression. Or consider, as Dr. Patil suggested yesterday, the multivariate distributions that may be at work to produce the phenomena we see as marginal distributions. (2)Looking at pairwise isoboles: again, we're bound by the paper plane. What about colors, faces, perspective, etc. It's almost ten years since Gnanadesikan published his book on ways to look at multivariate events. Let's consider other graphic devices, enlist the computer. (3)Looking at complex biological systems: while the organisms whose risk concerns us will almost always be complex, whether as a human or as an ecosystem, perhaps we can find other indicators of the likely response. More work needs to be put into examining and developing short term assay surrogates for prediction.

In summary, in this paper, a narrowly defined response addition

$$R(x_1,x_2,T) = x_1F_1(T) + x_2F_2(T) + E(x_1,x_2,T),$$

where $E(x_1,x_2,T) = 0$ for any x_1, x_2, T such that $x_1 + x_2 = 1$, namely, the special case of linear responses at fixed concentrations, permits (1)the construction of a test statistic and (2)the use of short term data. Furthermore, it calls attention to the literature that uses both composition and concentration to examine the behavior of mixtures.

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Table 1

Purpose	provide risk estimates from joint exposure	elucidate mechanisms of joint toxicity
Situation	assume components are unknown; predict curve at dose not studied	assume components are known; decide if joint action at dose studied
Methods need	procedures robust against misspecification	tests of full *vs* reduced models

STATISTICAL DIRECTIONS TO ASSESS EFFECTS OF COMBINED TOXICANTS

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ABSTRACT

The papers presented at the ASA/EPA Conference on Current Assessment of Combined Toxicant Effects are discussed. The papers illustrate the existence of screening methodologies to indicate when "interaction" between toxicants is likely. This can help assess mixtures toxicity for mixtures of a small number of toxicants at dose levels in the experimental range, but additional methods need be developed when extrapolation from one dose level to another is required or when more complex mixtures are assessed. The conference provided some limited guidance on the use of models for such cases, but greater statistical efforts are needed.

KEYWORDS

Joint Action Models
Complex Mixtures
Combined Toxicant Effects
Interaction

1. INTRODUCTION

This paper attempts to summarize the use of statistics to address the toxicity of mixtures and to suggest alternative statistical approaches that might be taken to achieve further progress in addressing the issue. Emphasis is given to the papers presented at this conference.

The toxicity of mixtures is clearly an important subject. If there were only 100 potentially toxic agents, the possibility of unusual or unexpected combined effects is hardly trivial. Taking combinations of two agents at a time, the matrix of combinations yield 4950 cells. If the probability of one agent influencing the toxicity of another were even as low as 0.01, there would still be 49 combinations where the toxicity of the combined toxicants would be different from the sum of the toxicity of the individual toxicants in assessing combined toxicant effects. The real world of thousands of agents and mixtures, far more complex than binary, obviously has considerable potential for a large number of "interactive" effects.

One of the problems in assessing combined toxicant effects is that there is a whole range of issues to be resolved. Mixtures can be defined at different levels of complexity. Much of the research to date and of the research reported here has been performed with binary mixtures. This is probably due to two reasons. First of all, as Kodell (1986) pointed out in his introduction, the earliest work was performed with drugs and pesticides, the objective being to examine the effectiveness of one of these substances in the presence of another. Hence only simple combinations were studied. (The simultaneous presence of environmental and other agents was ignored or assumed to be unimportant.)

Secondly, binary substances are a conceptual aid. The best approach for understanding a mixture's toxicity profile is to consider simple mixtures first. This can provide insights on how to analyze more complex mixtures, which realistically reflect exposure. Environments are complex; pure mixtures do not exist. We do not inhale, ingest or absorb pure substances or even a handful of substances, but mixtures of numerous substances. If, perchance, exposure were to be pure, the purity would cease once the substances entered the bloodstream. One potential approach to assess mixture toxicity is to divide the mixture into its components and to study these singly and in combination to arrive somehow at an estimate of the mixture's toxicity. Often, however, the mixture is ill-defined; its components cannot be defined. In such circumstances, one can only work with the total mixture and/or its fractions. Binary experiments are still possible, but assume a different role here as the experimental agents may be mixtures themselves.

Time complicates the definition of a mixture. Mixtures and exposures thereto can vary considerably over time, and this variation can influence the mixture's toxicity. Thorsland and Charnley (1986) show the temporal importance of cigarette smoking in a mixture with another carcinogen. In reality, human exposure patterns are even more complex, and it will be necessary to estimate and characterize this time variability and to determine its influence.

2. OBJECTIVES OF RESEARCH

Another problem associated with current toxicity assessment approaches is that many questions are asked of mixtures, and different approaches are appropriate for different questions. The questions will dictate the research objectives and corresponding statistical tools.

The most commonly asked questions probably relate to the three given below.

1. Under ambient conditions, is the mixture hazardous?
    a. Is interaction likely to occur? How is it defined?
    b. What is the dose-response surface for the mixture?
2. How toxic is the mixture compared to other mixtures? Other substances? Are similar mixtures equally toxic?
3. What is (are) the toxic component(s) of the mixture?

The papers at this conference address the first question with most of them focusing on question 1a, although the specific questions addressed are variations of the question. Several such as Weber et al. (1986) ask whether a given joint action model fits a data set. Machado (1986) and Chen et al. (1986) explicitly ask question 1a as to whether interaction and dose additivity exist. Other papers examine the presence of interaction over a broader range of dose-exposure levels and hence try to describe a dose-response surface. Thorsland and Charnley

(1986) address the toxicity of mixtures over dose ranges where extrapolation models are required. Christensen (1986) considers the issue but his objective is different. Very low doses (and hence extrapolation) are of lesser concern for fish than for humans, where risks to individuals of 10^{-5} or less are of policy concern.

The collection of papers suggests that question 1a can be answered for simple mixtures of two to three substances. A response to this question for more complex mixtures is hampered by unwieldy experimental designs and unrealistic data requirements. This situation can be alleviated somewhat by fractional factorial designs although these were not explicitly discussed at the conference. Question 1a is important for screening purposes; answers to it can suggest where "interaction" is likely to be present. A caution, however, is that the presence or absence of "interaction" at one set of dose levels need not generally imply the same result for other dose levels. Thorsland and Charnley (1986), for example, show that conclusions derived at "high" dose levels may not be equally true at "low" dose levels. Experimental results suggest this as well. In a series of fire toxicology experiments, Levin and coworkers (1986) demonstrate a relatively complex "interactive" effect of CO and CO_2 on the mortality of rats. Over a part of the dose range, mortality response appears to increase with increasing CO_2 concentrations for a fixed CO level. The very opposite appears to occur at other CO levels. Hence, a conclusion based on experiments over a limited dose range could not be generalized correctly.

Most of the historical terminology problems so well described by Kodell (1986) relate to question 1a because definitions of "interaction" were tied to specific models. As we progress beyond this screening question towards questions 1b, 2, and 3, much of this confusion will be resolved.

3. GENERAL PROBLEMS IN ASSESSING MIXTURE TOXICITY

3.1 Information Availability

The nature of available information will obviously influence the approach for addressing the mixture's toxicity. Most of the conference papers assumed that it was possible to identify the components of the mixture. If the components are unknown, the approaches discussed here must be modified or replaced. This will be discussed in the next section.

3.2 Pharmacokinetics

Another important information question is that of pharmacokinetics. This issue was addressed by Feder (1986) in his discussion of Thorsland and Charnley (1986) and to a lesser extent by Weber et al. (1986) and others who undertook some studies of specific organ systems in an effort to achieve "better" model fits. Obviously, responses to a dose can be more accurately estimated if the dose is that at the site of biologic activity. Unfortunately, the "effective dose" often is not known and the "administered dose" is used in estimating the dose-response relationship. This is obviously less than optimal in the case of a simple toxic, but the situation becomes even more complex in the case of a mixture. For example, misinterpretation could arise if the relative composition of the mixture were to change as a result of chemical interactions or of differential absorption, distribution, metabolism, or elimination, which varied with dose or some other factor independent of the mixture. Current pharmacokinetic models attempt to describe the fate of a single chemical and do not treat complexities that mixtures can introduce. Such complexities can distort the estimated dose of a mixture at target sites, where toxicity effects are initiated. Pharmacokinetic assumptions about compositional changes in the mixture would also have to hold across all species involved in any extrapolation across species, otherwise the validity of such extrapolation would be in question. Given the importance of this issue, more attention to pharmacokinetics is clearly warranted in assessing the toxicity of mixtures. The development of both pharmacokinetic data and models for mixtures is needed.

4. EMPHASIS ON COMPONENTS

The papers at the conference considered synthetic approaches in which a mixture was constructed from limited (two or three) components. As indicated above, this emphasis requires that the mixture be simple and well-characterized. These requirements, particularly the former, are not always realistic. At issue is whether and how existing methods can be adapted to more complex and realistic situations.

The complexity issue can be addressed by extending the methods used to several variables beyond the two or three considered. In this regard, some of the methods are more amenable than others. Those methods that depend upon experimental designs are hampered by practical considerations. Toxicology experiments can rarely accept more than a limited number of combinations of substances, otherwise, they become too costly and uniform experimental conditions for all combinations become difficult to maintain.

Simple factorial designs clearly limit consideration of mixtures more complex than three or four substances, but fractional factorial designs can extend the complexity of mixtures studies considerably. For example, designs for a mixture of 15 components could be constructed which required only 52 treatment groups (for combinations of doses), yet would still allow estimation of the toxicity of all 15 substances singly and of pairs of six of the substances. A simple factorial design for this mixture would require 32,768 treatment groups.

Another approach to assessing the toxicity of more complex mixtures is given by Thorsland and Charnley (1986), namely, the use of a model to estimate toxicity. Their results suggest that for their model, toxicity at "low" doses is additive across components in the mixture, i.e., "interaction" effects become negligibly smaller as the dose level decreases. Under these results, mixtures of several known components can be easily addressed by adding the toxicity of the components. This requires, however, that the toxicity of the components be known.

When the components of a mixture are unknown, two approaches are possible. A mixture with unknown components can be fractionated into mutually exclusive mixtures; the resulting mixtures then can be analyzed as if they were single substances to estimate any "interaction" of the resulting mixtures. Such an approach has been considered to address the toxicity of unleaded gasoline, a very complex mixture whose constituents are not completely specified. (Feder et al. 1984).

When the components of the mixture are not known, a second approach is to study the mixture directly as has been done with cigarette smoke. If the mixture toxicity is of interest, it may not matter whether or not there is interaction among the mixture's components. The toxicity could be assessed for several mixtures in the same class (e.g., different brands of cigarettes, vapors from different gasolines or exhaust fumes from different diesel engines) to determine if the toxicity is relatively robust across the class of mixtures. Feder et al. (1984) discuss this approach as well.

Extrapolation of toxicity from high to low doses could introduce a problem with the latter approach. Extrapolation models have been developed for single substances, and their application to a mixture could cause problems. Consider the example given by Thorsland and Charnley (1986). They give in Table 1 a bioassay design which gives an interaction term large enough to double the risk over that predicted by additivity at low doses. Under that design, for x_1 at a level of 1.05415×10^{-5} the risk is about 1×10^{-5}. For x_2 at 2.10828×10^{-5}, the risk is also about 1×10^{-5}. Under an additive model, the risk of a mixture of x_1 and x_2 would be 2×10^{-5}, whereas the true model gives the risk of 4×10^{-5}. Now if only a mixture of x_1 and $x_2 = x_3$ were tested in a bioassay design, extrapolation from the high dose levels in Table 1 (4.8713×10^{-3} for x_1 and 9.7486×10^{-3} for x_2) would yield a risk estimate of about 3.9×10^{-4} for the mixture at the low dose level with true risk 4×10^{-5}, i.e., we would over-predict the mixture's toxicity by an order of magnitude. This result should be placed in perspective, however. Table 1 reflects an extreme example and a factor of ten may be reasonable given some of the other uncertainties inherent in similar risk assessment exercises.

5. MODEL DEPENDENCE

Models are a major topic of this conference. All of the papers assume some model in addressing mixture toxicity, although the complexity of models varies considerably from response and concentration additive models to Hewlett-Plackett, Ashford-Cobbey and multistage models. In some papers, the models are tested to determine if they are consistent with data. It is noteworthy that the data are not always consistent with a given model. In the case of the multistage model, there is no way to test the fit of the model in the "low" dose range. A model may not be appropriate and assumption of the wrong model can lead to incorrect inferences. Siemiatycki and Thomas (1981), as Kodell (1986) has pointed out, show that "data can be consistent with a particular model even though the underlying conditions...are not met." As a result of this, a fitted model may be incorrect and lead to incorrect inferences about interactive effects. Siemiatycki and Thomas (1981) illustrate this point well.

There are three alternatives to this problem. One is to apply several models and to place greatest confidence in those results where several models converge. Christenson (1986) applies several models to the same data. The models agree over a fairly wide range, but diverge considerably in the tails yielding considerable uncertainty about what happens there.

A second approach to this problem is to use the data to generate a dose-response surface. Chen et al. (1986) gives one approach to this problem. An alternative is that applied by O'Sullivan to the fire toxicology data of Levin et al. (1986). Using generalized linear models to estimate the toxicity of individual components and their combination from the experimental data. Given the availability of recent codes such as GLIM, these methods are relatively easy to apply, and require relatively few underlying assumptions. The principal drawback of this approach is the requirement of a large number of data points, considerably more than usually available from experimental data. Also, for this method it can be dangerous to extrapolate outside the range of the observed data because interactions among the mixtures components may be dose-dependent in some poorly understood manner.

Another way to avoid the use of a specific model relating toxicity to dose is to apply Bayesian methods to extrapolate between mixtures. The work of Harris (1983) and DuMouchel and Harris (1983) is instrumental here. They see the problem as one in combining experimental results.

Consider a collection of mixtures with y_i denoting the experimentally derived toxicity or some other property of mixture i; θ_i is the true measure of y_i for mixture i. The problem is to ascertain θ_j for another mixture j, using all available evidence. DuMouchel and Harris (1983) define $y_i = \theta_i + \varepsilon_i$ where ε_i is a measure of "within-experiment" error. X_i is a vector of characteristics for mixture i, such as physical-chemical characteristics, component data, or selective toxicity results. The authors then define a hypothetical common mechanism, f, that relates the θ's to X_i, namely, $y_i = \theta_i + \varepsilon_i = f(X_i, \beta) + \delta_i$, where β is a set of hyper-parameters and δ_i is the "error of imperfect relevance" between studies. For example, δ_i, could represent nonlinear interactions between elements in the mixture i, if f were a linear model. DuMouchel and Harris (1983) then develop and use prior distributions on the δ_i, the ε_i, and β, to estimate the posterior distribution of θ_i given data y.

An example of the above would be the estimate y_i of the carcinogenicity of a mixture i, such as unleaded gasoline:

$$y_i = \theta_i + \varepsilon_i = f\,(X_i,\ \beta) + \delta_i\ ,$$

where θ_i is the true carcinogenicity of the mixture, ε_i is some error associated with the measure of y_i such as the error due to extrapolating from rats to humans or the error associated with a short-term test. The β could be a vector of the toxicities of the major components of gasoline and X_i is the vector of concentrations of the components. For example, $f(X_i,\beta) = X_i\beta$, the model could be interpreted as an additive model under which the toxicity of the mixture is the sum of the toxicities of its constituents. In this case, δ_i is a measure of interactions among the mixture constituents. Given prior distributions on δ_i, ε_i, and β, one can estimate the posterior distribution of the mixture toxicity given the data y. One can extend the context here by defining ε_i such that $y_i = \theta_i' + \varepsilon_i'$, where θ_i' represents another mixture for which no observed data are available. Other extensions are possible. See Harris (1983) and DuMouchel and Harris (1983).

6. CONCLUSIONS

The papers at this conference suggest that statistics to date has concentrated upon the problem of whether "interaction" exists and how it can be characterized. In this area, we have made considerable progress. We now have valuable screening tools that indicate when interactions may be important. Now, we need to ask more specific questions such as how important the interactions are at doses that may be different from those in the experiments where "interaction" is measured. Interpolation and extrapolation are required. These are roles for models that attempt to describe quantitatively the complex biology or toxicology of mixtures.

Models provide a means to describe and summarize experimental results and to relate them to underlying biology, but models for mixtures are in their infancy. A research priority is the development of improved models to address mixture toxicity. Thorsland and Charnley (1986) provide an important example of the direction that such models can take.

Models are imperfect tools. As such, they have limits. At best, they reflect the limits of biological knowledge. Models also delve into the unknown and unknowable when addressing such issues as high-to-low dose extrapolation. In these areas, models may be the only available tool, but their results are subject to considerable uncertainty, a greater uncertainty than they may imply. The limits and uncertainties of models need to be stated as part of their use.

In reality, modeling efforts often lag behind biological developments. Hence, one way to improve models is to achieve greater understanding of biological mechanisms. Biological intuition also can help direct modeling and statistical approaches. Weber et al. (1985), for example, help identify greater needs by following their intuition to illustrate the poor behavior of zinc-nickel interactions in the context of simple models.

Models and statistics support the major strategies to assess mixture toxicity, but models and statistics are only one criterion for development of strategies. Pragmatism and biology are foremost considerations. Pragmatically, it is not possible to test every combination of substances in every mixture. The challenge before us is to use statistics to move away from this approach towards one that is consistent with biology.

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APPENDIX A: ASA/EPA Conference on Current Assessment of Combined Toxicant Effects Program

ASA/EPA CONFERENCE
ON CURRENT ASSESSMENT OF
COMBINED TOXICANT EFFECTS

CAPITOL HOLIDAY INN
WASHINGTON, D. C.

MAY 5-6, 1986

THIS CONFERENCE IS BEING SUPPORTED BY EPA ASSISTANCE AGREEMENT NO. CX-813005-01-0, WITH COOPERATION FROM THE AMERICAN STATISTICAL ASSOCIATION.

ASA/EPA CONFERENCE ON CURRENT ASSESSMENT OF COMBINED TOXICANT EFFECTS
CAPITOL HOLIDAY INN, WASHINGTON, D.C.
MAY 5-6, 1986

Conference Chair: **Emanuel Landau**
American Public Health Association

Monday, May 5

8:30 - 9:00 AM	Welcome
9:00 - 10:00	**Ralph L. Kodell**, National Center for Toxicological Research Keynote: Modeling the Joint Action of Toxicants: Basic Concepts & Approaches
10:00 - 10:15	OPEN DISCUSSION
10:15 - 10:45	BREAK
10:45 - 11:30	**Todd Thorslund**, ICF/Clement Use of the Multistage Model to Predict the Carcinogenic Response Associated with Time-Dependent Exposures to Multiple Agents
11:30 - 12:00 NOON	Discussant: **Paul I. Feder**, Battelle Columbus Labs
12:00 - 1:15 PM	LUNCH
1:15 - 1:45	**Stella G. Machado**, Science Applications International Corporation Assessment of Interaction in Long-Term Experiments
1:45 - 2:15	Discussant: **Chao Chen**, Carcinogen Assessment Group, EPA
2:15 - 2:30	OPEN DISCUSSION
2:30 - 2:45	BREAK
2:45 - 3:15	**Lavern J. Weber**, Mark Hatfield Marine Science Center Concentration and Response Addition of Mixtures of Toxicants Using Lethality, Growth, and Organ System Studies
3:15 - 3:45	Discussant: **Bertram D. Litt**, Office of Pesticides, EPA
3:45 - 4:15	OPEN DISCUSSION
5:00 - 6:30	RECEPTION

Tuesday, May 6

9:00 - 9:30 AM	**Steven J. Broderius**, EPA Environmental Research Laboratory, Duluth Joint Aquatic Toxicity of Chemical Mixtures and Structure-Toxicity Relationships
9:30 - 10:00	Discussant: **G. P. Patil**, Center for Statistical Ecology and Environmental Statistics, Penn State University
10:00 - 10:15	OPEN DISCUSSION
10:15 - 10:30	BREAK
10:30 - 11:00	**Eric R. Christensen**, University of Wisconsin-Milwaukee Development of Models for Combined Toxicant Effects
11:00 - 11:30	Discussant: **Richard C. Hertzberg**, EPA
11:30 - 11:45	OPEN DISCUSSION
11:45 - 1:15 PM	LUNCH
1:15 - 1:45	**James J. Chen**, National Center for Toxicological Research, FDA A Response-Additive Model for Assessing the Joint Action of Mixtures
1:45 - 2:15	Discussant: **Elizabeth H. Margosches**, EPA
2:15 - 2:30	BREAK
2:30 - 3:30	Summary of Conference: **Ronald Wyzga**, Electric Power Research Institute
3:30 - 4:00	OPEN DISCUSSION

APPENDIX B: Conference Participants

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May 5-6, 1986
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